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# *Scytalidium uredinicola* and the Control of Western Gall Rust

BY

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(C)

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled SCYTALIDIUM UREDINICOLA AND THE CONTROL OF WESTERN GALL RUST submitted by James E. Cunningham in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The purpose of this study was to ascertain whether the natural antagonism of Scytalidium uredinicola against Endocronartium harknessii can be exploited to control outbreaks of E. harknessii, the western gall rust. This study focussed on two principal areas. One was the isolation and testing of S. uredinicola metabolites as natural fungicides. The second was the characterization of S. uredinicola submerged, batch cultures, as preliminary research leading to the production of mycelium or metabolites as biocontrol or chemical control agents.

A spore germination inhibitor was isolated from solvent extracts of S. uredinicola cultures by column chromatography, and it was identified as maltol (3-hydroxy-2-methyl-4H-pyran-4-one). Maltol was weakly inhibitory to the mycelial growth of various wood-decay fungi, but was more active as an inhibitor of E. harknessii spore germination. Maltol was tested as a systemic fungicide by using lodgepole pine seedlings artificially inoculated with E. harknessii. The application of maltol to the planting soil reduced the rate of infection by E. harknessii, with no apparent effects on the seedlings.

Certain trends in carbon and nitrogen metabolism occurred in submerged, batch cultures of S. uredinicola and appeared to be specific for total mycelial mass. Culture growth rate was significantly affected by inoculum size.

The time course of maltol concentration in the medium was correlated with the growth curve, and therefore was also affected. Hyphal growth of S. uredinicola was examined using phase-contrast microscopy, and the growth of S. uredinicola mycelium in submerged culture was measured by dry weight increase. It was concluded that the accelerating growth phase was best described by a non-logarithmic model which is dependant on inoculum size.

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TABLE OF CONTENTS


| CHAPTER | | PAGE |
|---|---|---|
| I. | INTRODUCTION | 1 |
| II. | LITERATURE REVIEW | 6 |
| | *Endocronartium harknessii* | 6 |
| | *Scytalidium* | 12 |
| | Fungal Spore Germination | 16 |
| | Kinetics of Fungal Growth | 18 |
| III. | MATERIALS AND METHODS | 25 |
| | Microorganisms | 25 |
| | Production of *S. uredinicola* Metabolites | 28 |
| | 1) Culture procedure | 28 |
| | 2) Extraction of crude metabolites | 28 |
| | 3) Isolation of the spore germination inhibitor | 29 |
| | Bioassay Systems | 29 |
| | 1) Spore germination assays | 29 |
| | 2) Mycelial growth assays | 30 |
| | 3) Yeast MIC determinations | 31 |
| | Analytical Procedures | 31 |
| | 1) Thin layer chromatography | 31 |
| | 2) Gas chromatography | 31 |
| | 3) Ultraviolet absorbance scanning | 32 |
| | Growth Curves | 32 |
| | 1) Inoculation technique for synthetic medium cultures | 32 |

2) Dry weight determination ..... 33
3) Determination of maltol concentration in growth media ..... 33
4) Glucose determination ..... 33
5) Total carbohydrate ..... 34
6) Ammonium determination ..... 34
7) Nitrate determination ..... 34
Measurement of Hyphal Growth ..... 35
*In Vivo* Testing of Maltol as a Fungistatic Agent in Pine Seedlings ..... 35
1) Growth of pine seedlings ..... 35
2) Application of Maltol ..... 36
3) Inoculation with *E. harknessii* spores ..... 36
IV. RESULTS ..... 37
Culture Conditions and Inhibitor Production 37
Inhibitor Purification and Identification . 43
1) Mobility of Inhibitory Substances on TLC ..... 43
2) Isolation of the Inhibitory Metabolite by Column Chromatography 49
Identification of the Inhibitory Metabolite from *S. uredinicola* ..... 63
Other Metabolites of *S. uredinicola* ..... 70
*S. uredinicola* Growth Kinetics, Carbon and Nitrogen Utilization, and Maltol Production 76

1) Effect of inoculum size on growth, pH, and maltol production in malt extract broth ..... 76
2) Examination of hyphal growth ..... 77
3) Culture growth rate in malt extract broth ..... 90
4) Effects of inoculum size on synthetic medium cultures ..... 104
Biological Effects of Maltol ..... 133
1) Conditions for E. harknessii spore germination ..... 133
2) Effect of maltol and other compounds on E. harknessii germination ..... 136
3) Maltol as an inhibitor of mycelial growth ..... 144
4) Survey of yeast sensitivities to maltol ..... 144
In Vivo Protection of Pine Seedlings with Maltol ..... 144
Presence of Maltol in Lodgepole Pine ..... 160
V. DISCUSSION ..... 161
Culture Conditions and Inhibitor Production 161
Inhibitor Purification and Isolation ..... 165
Growth Kinetics of S. uredinicola ..... 168
Carbon and Nitrogen Utilization in S.

*uredinicola* Cultures ..... 177

Biological Effects of Maltol ..... 182

VI. CONCLUSIONS ..... 185

VII. REFERENCES ..... 188

APPENDIX A ..... 206

APPENDIX B ..... 211

APPENDIX C ..... 212

APPENDIX D ..... 215

LIST OF TABLES


| Table | Description | Page |
|---|---|---|
| 1 | A summary of metabolites isolated from *Scytalidium* species ..... | 14 |
| 2 | Comparison of the models for growth of *S. uredinicola* in submerged culture ..... | 101 |
| 3 | The activity of maltol against the mycelial growth of various wood decay fungi in submerged culture ..... | 147 |
| 4 | MIC of maltol against 46 yeast isolates ..... | 149 |
| 5 | The effect of maltol on pine seedling susceptibility to infection by *E. harknessii* . | 159 |
| 6 | A comparison of four growth functions fitted to a hypothetical model ($L_t$) ..... | 173 |
| 7 | Plants from which maltol has been isolated ... | 207 |

LIST OF FIGURES


| Figure | Description | Page |
|---|---|---|
| 1 | The collection of E. harknessii spores from lodgepole pine ..... | 27 |
| 2 | The effect of inoculum size on culture growth rate and mycelial yield ..... | 39 |
| 3 | The relation of maltol concentration, medium pH, and mycelial yield with time in submerged cultures of S. uredinicola 4149 ..... | 41 |
| 4 | The distribution of sporostatic activity on a preparative thin layer chromatogram ..... | 45 |
| 5 | Photomicrographs of abnormal spore germination caused by S. uredinicola metabolites recovered from preparative thin layer chromatography ... | 47 |
| 6 | Thin layer chromatographic analysis of crude extract fractions obtained by silica gel column chromatography ..... | 52 |
| 7 | Gas chromatographic analysis of fraction 21 from chromatography column I of the inhibitor purification procedure ..... | 54 |
| 8 | Photograph of Chromatography column II in the inhibitor purification procedure ..... | 56 |
| 9 | The elution profiles of maltol and benzeneacetic acid from column III of the inhibitor purification procedure ..... | 59 |

| Figure | Description | Page |
|---|---|---|
| 10 | The elution profiles of maltol and benzeneacetic acid from column IV of the inhibitor purification procedure ..... | 62 |
| 11 | The structural formula of maltol ..... | 65 |
| 12 | Mass spectra of the purified, sporostatic metabolite from S. uredinicola and its computer-library match ..... | 67 |
| 13 | UV absorbance scans of the purified, sporostatic metabolite of S. uredinicola and an authentic sample of maltol ..... | 69 |
| 14 | The mass spectrum of an unidentified S. uredinicola metabolite ..... | 72 |
| 15 | The UV spectrum of an unidentified S. uredinicola metabolite ..... | 72 |
| 16 | Mass specra of a purified, S. uredinicola metabolite and its computer-library match, benzeneacetic acid ..... | 75 |
| 17 | The effect of inoculum size on maltol production, pH, and mycelial growth in S. uredinicola 4149 malt extract broth cultures . | 78 |
| 18 | Photomicrograph of a typical S. uredinicola 4149 inoculum preparation ..... | 82 |
| 19 | The extension of individual hyphae from a single spore, and their combined rates ..... | 84 |

| Figure | Description | Page |
|---|---|---|
| 20 | Photomicrograph showing the variability of outgrowth from individual spores of S. uredinicola 4149 ..... | 86 |
| 21 | The extension rate of the main axis of an S. uredinicola hypha at a colony margin ..... | 89 |
| 22 | The extension rates of an S. uredinicola hyphal branching system at a colony margin ... | 92 |
| 23 | Photomicrographs of the hyphal branching system examined in Figure 22 ..... | 94 |
| 24 | A schematic depiction of the hyphal branching system described in Figures 22 and 23 ..... | 97 |
| 25 | The early growth phase of S. uredinicola 4149 in malt extract broth ..... | 100 |
| 26 | Logarithmic, cube root, and sixth root transformations of S. uredinicola 4149 growth in malt extract broth cultures ..... | 103 |
| 27 | Schematic depiction comparing equal total lengths of hyphae generated from different inoculum sizes ..... | 106 |
| 28 | Photomicrographs of S. uredinicola mycelium from synthetic medium, submerged cultures .... | 108 |
| 29 | The final appearance of mycelium from large and small inoculum cultures ..... | 112 |

| Figure | Description | Page |
|---|---|---|
| 30 | Graph showing new mycelium formed within time intervals, for large and small-inoculum cultures | 116 |
| 31 | The relationship between new mycelial mass and inoculum size | 118 |
| 32 | The ratio of carbohydrate to nitrogen removed from synthetic medium by S. uredinicola 4149 as a function of inoculum size | 121 |
| 33 | Growth curves of small and large-inoculum cultures showing pH, medium glucose, medium ammonium, and dry weight | 123 |
| 34 | The ratio of glucose to ammonium used by small and large-inoculum cultures as a function of culture age, and of total new mycelium | 126 |
| 35 | The time interval separating the growth curves of small and large-inoculum cultures | 129 |
| 36 | Maltol production by S. uredinicola in synthetic medium | 132 |
| 37 | The response of E. harknessii germination to pH | 135 |
| 38 | The response of E. harknessii spore germination to phosphate concentration | 135 |
| 39 | The dose-response of E. harknessii spore germination to maltol | 138 |

| Figure | Description | Page |
|---|---|---|
| 40 | The effect of glutamic acid on E. harknessii spore germination in the presence of maltol .. | 140 |
| 41 | The dose-response of E. harknessii spore germination to rhodizonic acid ..... | 143 |
| 42 | The dose-response of E. harknessii spore germination to 2,3-dihydroxybenzoic acid ..... | 143 |
| 43 | The dose-response of Ceratocystis ulmi to maltol ..... | 146 |
| 44 | The distribution of MIC of maltol between yeast isolates ..... | 153 |
| 45 | A comparison of the MIC frequency distribution for two yeast isolates ..... | 155 |
| 46 | Photograph of a typical lodgepole pine seedling at the age when inoculated with E. harknessii, showing the extent of its root system ..... | 158 |
| 47 | A hypothetical model of the growth of a hyphal branching system ..... | 171 |

# I. INTRODUCTION

Intensive management, including reforestation practises, is of increasing importance in western Canadian forest industries. The production of nursery stock and the amount of planted stock is therefore increasing to satisfy provincially imposed standards of forest management. Consequently the disease problems of seedlings and young managed stands, which differ from those of natural stands, are commanding greater attention. One of the diseases that has become significant in the reforestation process is the pine stem rust Endocronartium harknessii (J.P. Moore) Y. Hiratsuka.

This study was undertaken in response to the need for an effective agent to suppress outbreaks of the rust fungus E. harknessii in nursery, tree farm, or forest situations. Conventional fungicides applied to these situations suffer the shortcomings of providing only temporary control, failing to eliminate the source of further infection since they do not act systemically (Merril and Kistler 1976), and frequently they are undesirable becouse of their accumulation in the environment. Alternative control measures that have been examined include the use of antibiotic substances (Hinds and Peterson 1966), and biological control agents (Tsuneda and Hiratsuka 1981); however, neither has shown promising experimental results. Nonetheless, an examination of the parasitic mechanisms

which occur naturally may generate new approaches in the development of more effective methods. Accordingly, the observations of fungitoxic metabolite production by a natural mycoparasite of E. harknessii (Fairbairn et al. 1983, Tsuneda et al. 1980) provided the foundation for this study.

E. harknessii, known commonly as western gall rust, is among the most destructive fungal diseases of pines in North America. Hosts of this pathogen are: Pinus banksiana Lamb (jack pine); P. contorta Dougl. (lodgepole and shore pine); P. mugo Turra (mugo or Swiss mountain pine); P. muricata D. don (bishop pine); P. nigra Arnold (Austrian or Corsican pine); P. pinaster Ait. (maritime pine); P. ponderosa Laws (ponderosa pine); P. radiata D. Don (Monterey pine); and P. sylvestris L. (Scotch pine). The large, cankerous galls resulting from E. harknessii infection slowly girdle and destroy affected pine stems. Aecioid teliospores, seen as a bright orange spore mass which is borne seasonally on the gall, may directly infect or reinfect pine tree hosts. The need for effective control of severe rust outbreaks arises not only from the loss of nursery and plantation stocks, but also from the devaluation of natural stands (Hiratsuka and Powell 1976).

Three fungi are frequently observed on the rust galls and are significantly antagonistic towards E. harknessii. These organisms are Scytalidium uredinicola Kuhlman et al.,

Cladosporium gallicola Sutton, and Monocillium nordinii (Bourchier) W. Gams. The mechanism of antagonism differs between the mycoparasites, and for purposes of biological control it has been considered that the activities of S. uredinicola and C. gallicola are complimentary. their combined application may effectively limit the spread of western gall rust (Tsuneda and Hiratsuka 1981).

The effect of S. uredinicola on E. harknessii in wood tissue suggested that a diffusible metabolite of S. uredinicola is a significant aspect of the gall inactivation arising from this hyperparasitism (Tsuneda et al. 1980). Subsequently, inhibition of E. harknessii spore germination by S. uredinicola culture filtrates, as well as by solvent extracts of these filtrates, was shown (Fairbairn et al. 1983). This indicated that an ecologically favorable control agent might be found by examining metabolites of S. uredinicola. From the influence of culture conditions on inhibitor production by S. uredinicola (Fairbairn et al. 1983), it is apparent that the growth characteristics of this organism in submerged culture must be better understood if the laboratory observations are to be related to the in situ antagonism.

A fundamental difference between fungal growth on solid media as opposed to agitated, submerged cultures is that some degree of metabolic phasing may be achieved in the latter (Bu'Lock et al. 1965). On agar plates, a gradient of

nutrient depletion and metabolite accumulation from the center of a colony is established. All stages of mycelial development, from rapidly elongating hyphae at colony margins to differentiated and conidiogenous cells at colony centers, are present. This provides a closer approximation of *in situ* growth than do submerged cultures. Fungal hyphae in submerged cultures are exposed to conditions made homogeneous by agitation. consequently the medium supports rapid growth until nutrient depletion or other changes cause a shift in metabolism; a separation of the growth phase from the conidiogenous phase may occur. It is therefore of interest to learn what factors will affect the kinetics of culture growth so that cultures may be replicated accurately, and to identify the phase of hyperparasite growth which is associated with its activity against *E. harknessii*.

There were four main directives in this investigation:

1) The isolation of the *S. uredinicola* metabolite that inhibits *E. harknessii* spore germination, from the solvent extracts of *S. uredinicola* cultures described by Fairbairn *et al.* (1983).

2) The investigation of inhibitor production by submerged cultures of *S. uredinicola*.

3) The investigation of the kinetics and nutrition of *S. uredinicola* growth in submerged cultures. The goals of these experiments were culture reproducibility and to assess

the potential of S. uredinicola as a biological control agent.

4) The evaluation of the germination inhibitor from S. uredinicola as a control agent against some other phytopathogenic fungi.

## II. LITERATURE REVIEW

### Endocronartium harknessii

The earliest record of western gall rust was published by J.P. Moore in 1876 (Peterson 1967), where it was described under the name Peridermium harknessii. Owing to the complexity of most rust life cycles, considerable confusion surrounded the identification of the western gall rust organism as to whether it was distinct from similar peridermium rusts on other hosts. This point is illustrated by a statement from Hedgecock (1911): "Peridermium harknessii resembles P. cerebrum so closely that all that is required to prove the identity of the two species is proof that the former has Cronartium quercum [the alternate form of P. cerebrum which occurs on Quercus sp.] for its alternate form." The author's ensuing attempts to prove this point experimentally were destined to failure. Similar experimental findings by Meinecke (1916) led that author to the conclusion of facultative heteroecism. P. harknessii was eventually recognized as a distinctive, autoecious organism, and it was reclassified into a new genus, Endocronartium (indicating an endocyclic life cycle), by Hiratsuka (1969). The possibility that E. harknessii is manifest in only the one form, vis western gall rust, dates back as far as Fromme (1916); he suggested that P. harknessii be regarded as autoecious until proven facultatively heteroecious. Such debate pervades the

literature to as recently as 1971. Peterson (1971) submitted the lack of correlation between outbreaks of C. ribicola and P. harknessii to be evidence of a distinctive life cycle, but he also stated that the possibility of Cronartium coleosporioides being an alternate form of P. harknessii was yet defensible. Presently, it is generally accepted that E. harknessii is a distinctive, autoecious pine stem rust, with a single sporulating state viz aecioid teliospores. A spermagonial state, however, is occasionally observed. Unlike the blister rust Cronartium comandrae, E. harknessii canker development on host trunks proceeds laterally at a slow rate, the consequence being low incidence of host mortality. The lumber value of an affected stand is reduced principally by cull and growth reduction resulting from trunk cankers. Such cankers reduce trunk strength, increasing the frequency of wind breakage (Peterson 1960, Baranyay and Stevenson 1964). A mathematical model has been advanced by Peterson (1960) to predict the time required for the girdling of a trunk by E. harknessii, based on the host and parasite growth rates. Essentially, young stem leaders may be girdled within three or four seasons, whereas gall development may be insufficient to girdle larger trees. The slow lateral advance of the canker accounts for the observation of viable rust mycelium as old as 200 years. Trees with slowly developing cankers serve as a perennial source of inoculum

for further rust infections, while prolonging the depressed wood value of the affected area.

Outbreaks of E. harknessii are usually confined to a few acres and cause minimal tree mortality. Some extreme values have been reported, ranging as high as 100% infection with galls occurring on 25% to 75% of the branches on each tree. Despite the low mortality rate, it is apparent that growth loss is inevitable (Baranyay et al. 1962, Baranyay and Stevenson 1964, Hiratsuka and Powell 1976). While numerous biological and abiotic factors may influence the frequency and severity of outbreaks, a wave-year phenomenon is known to occur and is likely attributable to meteorological trends (Meyer et al. 1982). Peterson (1960) indicated that over vast land areas a stable community may exist wherein a balance between regions of high and low gall rust incidence occurs. In this regard, it is noteworthy that infection rates may be elevated in manipulated stands (Ives et al. 1971). Western gall rust outbreaks in nursery and plantation situations present the most immediate concern, because of the high mortality observed with young trees and the possibility of dissemination by plants leaving a nursery. Suggested preventative measures include isolation of nurseries from native hosts, pruning or clear-cutting to remove spore producing galls, and fungicide application to prevent infection (Carlson 1969).

Control measures directed against pine stem rusts may

be silvicultural, chemical, biological (hyperparasitic agents), or genetical (breeding for host resistance). The pruning of E. harknessii galls from host limbs will curtail damage to the individual host, as well as prevent rust dissemination; however, this practise may not prove economically expedient. Moreover, there remains the problem of incipient infection in nursery stocks destined for outplanting. It has been recomended that nursery stock be located at a minimum distance of 300 yards from natural stands of E. harknessii hosts which may be a source of infection (Carlson 1969). Satisfactory protection of nursery pines can be achieved by spraying with conventional fungicides during the sporulation season. Bordeaux mixture (copper sulfate and calcium oxide), lime-sulfur, Ferbam (ferric dimethyldithiocarbamate), Ziram (zinc dimethyldithiocarbamate), Zineb (zinc ethylene bis dithiocarbamate), and nickel chloride are all known to be effective against pine stem rusts. Systemic control, desired to eradicate the source of inoculum, has not been achieved with conventional fungicides (Hiratsuka and Powell 1976). In a study on Scots pines 1.0 to 1.5 m tall, a single application of Maneb during sporulation season gave significant protection from E. harknessii infection, but three systemic fungicides (Benomyl, Oxycarbin, and Dowco 261) had no effect on spore production by active cankers (Merril and Kistler 1976). The antibiotic substances

cycloheximide (actidione) and phyloactin, shown to be effective against white pine blister rust (Moss et al. 1960, Moss 1961), were tested against E. harknessii on lodgepole and ponderosa pines. Temporary inhibition of canker development in the spray zone (lower 3½ feet of trunk) was noted; however, systemic activity was not apparent, and sporulation above the spray zone was not affected (Hinds et al. 1966).

Of the many fungi and bacteria which may colonize rust cankers, three hyphomycetous fungi are known to be directly parasitic on E. harknessii. These organisms are Monocillium nordinii, Cladosporium gallicola, and S. uredinicola. The principal action of M. nordinii on E. harknessii resides in the production of the antibiotic substance monorden (also called radicicol) and a group of five related compounds described as monocillins. Growth of M. nordinii within the rust gall is limited, failing to cause complete inactivation. The effectiveness of the antibiotic products of M. nordinii as control agents is not yet known (Tsuneda and Hiratsuka 1980, Ayer et al. 1980). C. gallicola colonizes the rust sorus during sporulation and covers the entire gall surface within days. The mode of parasitism involves penetration of the rust spores and absorption of the host cytoplasm. Although a substantial reduction of inoculum potential results, the activity of C. gallicola is restricted to the gall surface, and pathogenesis by E. harknessii is not

arrested (Tsuneda and Hiratsuka 1981). Conversely, by slow but pervasive growth throughout the sorus, S. uredinicola completely inactivates the rust gall after several seasons growth. Tsuneda et al. (1980) observed S. uredinicola causing degradation of rust spore walls and reducing spore viability, without penetrating the spores. Also, S. uredinicola penetrated into the wood below the gall and inhibited the growth of rust hyphae, apparently through the production of a diffusible, toxic metabolite. It has been suggested that the dissimilar parasitic processes of C. gallicola and S. uredinicola may be complementary as biological control agents: rapid reduction of inoculum potential by C. gallicola combined with ultimate gall inactivation by S. uredinicola (Tsuneda and Hiratsuka 1981).

In order to study the effects of antifungal metabolites as protectants against rust infection, an in vivo system which provides reliable detection within the shortest time possible is desirable. Allen and Hiratsuka (1985) describe the use of young lodgepole pine seedlings in which a high rate of infection may be achieved. Infection is detectable by a pigmentation symptom within ten days of inoculation, and gall development becomes evident within two months. A detailed account of the early stages of infection and host response was recorded (Allen and Hiratsuka 1985).

Scytalidium

Until recently, the taxonomy of hyphomycetous (imperfect) fungi has been based almost exclusively on thallic ontogeny, pigmentation, and morphological description. Without the systematic use of biochemical, nutritional, or other discriminating characteristics, the relationship between species within a form-genus is unclear (Hawksworth 1984, Kurtzman 1984). Indeed, the form taxa are designed for the purpose of identifying isolates by empirical criteria, and not with the intent to group organisms by their degree of relatedness (Moore-Landecker 1982). For these reasons, considerable caution should be exercised where comparisons between species of Scytalidium might be made.

S. uredinicola is the most recent addition to the form-genus Scytalidium Pesente. This form-genus is characterized by dematiaceous arthroconidia which have a diameter greater than the vegetative hyphae. The spores are formed intercalarly, or terminally on hyphae which cease to elongate upon conidiogensis (Sigler and Carmichael 1976). There are presently seven species of Scytalidium: S. album; S. aurantiacum; S. acidophilum; S. flavo-brunneum; S. uredinicola; and the Scytalidium state of Hendersonula toruloidea. Most of these species are known to be antagonistic against other fungi and/or produce antibiotic substances. A summary of metabolites isolated from

Scytalidium species is given in Table 1.

Scytalidium uredinicola was first isolated from the aecia of Cronartium fusiforme on Pinus taeda in North Carolina. A comparison of two rust outbreaks in separate counties led the authors to infer that aecial sporulation was not suppressed by S. uredinicola. The isolate was distinguished from other Scytalidium species by its habitat and arthrospore character (Kuhlman et al. 1976). In 1979 Hiratsuka et al. published the first record of S. uredinicola occurring in Canada; it was isolated from E. harknessii galls on P. contorta and P. banksiana. The authors reported reduced viability of rust spores from the hyperparasitized galls, and thereby indicated that S. uredinicola was a potential, biological control agent.

S. aurantiacum (isolated from pulpwood) and S. album (isolated from Norway spruce) were first described by Klingstrom and Beyer (1965). Both organisms were found to be antagonistic towards the conifer root rot fungus Fomes annosus. Thirty-eight isolates of Scytalidium from three species (S. album, S. aurantiacum, and S. lignicola) were screened, and the majority were found to produce fungitoxic substances (Klingstrom and Johanson 1972). The use of Scytalidium as a biocontrol agent against wood decay fungi was also investigated by Ricard and Bollen (1967). This followed their observation that a Scytalidium species often occurred in Douglas-fir poles which were free of decay. The

Table 1. A summary of metabolites isolated from *Scytalidium* species.

| Organism | Metabolite | Reference |
|---|---|---|
| *Scytalidium album* | scytalone | Findlay and Kwan 1973 |
| *Scytalidium album* | asterric acid | Stermitz *et al*. 1973 |
| *Scytalidium album* | 4,8-dihydroxytetralone | Findlay and Kwan 1973B |
| *Scytalidium album* | 5'-formyl-2'-hydroxyl-4'-methoxy-(E,E)-4-hexenophenone | Geigert *et al*. 1973 |
| *Scytalidium album* | 5'-formyl-2'-hydroxyl-4'-methoxy-(E,E)-sorbophenone | Geigert *et al*. 1973 |
| *Scytalidium album* | scytalidin | Strunz *et al*. 1972 |
| *Scytalidium flavo-brunneum* | various derivatives of 15-aza-24-homocholestadiene | Michel *et al*. 1975 |

identity of the species of Scytalidium isolated in this study was uncertain, and the designation FY strain was given. The isolate was later accepted as S. album (ATCC 16675). Again, the production of an antibiotic was implicated in the antagonistic activity. In field trials, Scytalidium was successfully established in Douglas-fir poles and prevented the development of Poria carbonica (which causes heartwood decay). The authors noted that marked variation in antibiotic production occurs between Scytalidium cultures, and that "thorough studies on the physiology of Scytalidium sp. are needed to solve the problems attendant in its use."

Regarding antibiotic production by S. album, a toxic substance was obtained in pure form and characterized by Klingstrom and Johanson (1972), but the chemical identity was not established. The biological properties reported were consistent with the compound scytalidin, but the physical data (mass spectrum m/e 527 molecular peak) were not. The chemical structure of the S. album metabolite scytalidin was elucidated independantly by Strunz et al. (1972), and Overeem and Mackor (1973). The latter publication described the compound as scytalidic acid, the major metabolite isolated from Scytalidium FY cultures, which had no toxicity against P. carbonica. Subsequently, in a thorough investigation of the antifungal properties of this compound, scytalidin was found to be a potent inhibitor of fungal growth at low pH but inocuous at neutral pH

(Stillwell et al. 1973). Although this substance shows no phytotoxicity at antifungal concentrations, its low solubility in water and pH dependance will likely limit its usefulness as a control agent. At least one other antifungal metabolite was present in the cultures from which scytalidin was isolated. This was shown by the activity of culture filtrate against Phytophthora infestans sporangia that were insensitive to scytalidin.

Fungal Spore Germination

Dormancy in fungal spores may be imposed by constitutive or exogenous controls. In general, the former may be self-inhibitors of germination or the requirement of an activating substance, while the latter are environmental conditions.

Self-inhibitors common to several rust fungi are methyl cis-3,4-dimethoxycinnamate and methyl cis-ferulate. These substances are highly potent inhibitors of germination and are easily washed from the spores. They were first detected by the crowding effect in which high density spore suspensions showed reduced germination (Foundin and Macko 1974). Other substances involved in the control of rust spore germination may be volatile substances detectable by reduced germination in closed vessels (Allen 1955), and self-stimulators which act in concert with self-inhibitors (Yarwood 1956). It is generally accepted that rust spores

have predetermined points (germ pores) at which germ tubes are initiated (Marchant 1979). Further possibilities for the control of germ tube emergence exist at this level, as demonstrated by the suppression of germination by inhibition of a protease required for germ tube initiation. This system was studied in macroconidia of the Ascomycete Microsporium gypseum: it was found that while the entire spore outgrowth process required several hydrolases acting on the spore coat, the initiating step appeared to require an alkaline protease activity. The activity of this enzyme was shown to be regulated by inorganic phosphate which inhibited activity and calcium ion which reduced the effect of phosphate (Page and Stock 1971, 1972, 1974). The concentration of phosphate causing a 50% reduction of protease activity was 44 mM. Reduction of rust spore germination by phosphate is also known to occur, as shown with Uromyces phaseoli (Bell and Daly 1962).

Endocronartium harknessii spores will germinate on distilled water, requiring no exogenous nutrients and without a preceding period of dormancy. Storage of spores results in decreased germination and slight changes in optimal germination conditions. Optimal conditions for E. harknessii germination were found to be 15°C to 20°C, at pH 6 to 7. Maximum percent germination as well as maximum germ tube length is achieved at about 24 hours incubation (Powell and Morf 1966). Distinguishing features of E. harknessii

spore outgrowth include straight, septate germ tubes of determinant length, and a small branch that often arises from the proximal cell of the germ tube. Also, E. harknessii spores produce only one germ tube in contrast to the related rust Cronartium coleosporioides Arth. which may form more than one (Powell and Morf 1966).

## Kinetics of Fungal Growth

The growth of fungal cultures occurs at a rate arising from a number of components which are poorly understood, often unique to a species or strain, and consequently appear to resist generalized analyses. While it is convenient to describe fungal growth in terms of the phases observed with cultures of unicellular organisms, binary fission is not observed in filamentous fungi; hence, exponential growth should not necessarily be anticipated (Mandels 1965).

Kinetic analyses of experimental data are required where the time course of growth must be controlled or reproduced. Examples of situations in which the growth habit of an organism warrants attention are nutritional studies and the adaptation of fungal fermentation systems to large scale vessels. The desire for a generalized mathematical model describing fungal growth in submerged culture led to the proposal that, since growth is unrestricted and proceding in three dimensions, mycelial spheres should form with a constant radial increase;

therefore, the cube root of the fungal mass should increase linearly with time. Experimental data comparing the growth of Neurospora crassa plotted as dry weight, log dry weight, and cube root dry weight were presented, demonstrating that the cube root relationship is maintained for a greater length of time than is the logarithmic relationship (Emerson 1950). This model, however, is founded on the premise that mycelial spheres of uniform density are formed.

The cube root model was applied to an analogous situation, the growth of pellet forming fungi, where approximately spherical mycelial aggregates of varying size are formed. The analysis of Polyporus versicolor and P. ostreatus culture growth indicated that, within the statistical limits of detection, the exponential and cube root models fit the data equally well. It was concluded that the exponential model may be preferable for mathematical simplicity and its relation to other biological systems (Carroad and Wilke 1977). In this investigation, however, the dissimilar effect of different initial mycelial mass on each model was not considered. Compounding the difficulties in establishing a general model is the influence of oxygen diffusion (into mycelial pellets), inoculum size, hyphal ageing, and cellular differentiation on experimental data.

The effect of inoculum size on culture growth rate and mycelial yield was examined closely by Taber (1957), and

and Ward and Colotelo (1960). Taber demonstrated the use of standardized inoculum to minimize variance in yield in the context of nutritional studies, and he revealed how radically conflicting results regarding substrate utilization (in this case, mannitol and glucose as carbon sources for Claviceps purpurea were compared) may arise through alteration of inoculum size. Ward and Colotelo elaborated on the effect of inoculum by relating inoculum culture age and inoculum quantity to mycelial yield. They observed a dramatic decrease in yield from mycelial inoculum taken from late growth phase as opposed to early growth phase. Presumably this effect arises in part from the increasing proportion of senescent mycelium in the inoculum, resulting in an effect equivalent to alteration of inoculum size. Increasing inoculum size was found to increase yield towards a maximum that was believed to be imposed by nutrient depletion. The exact relationship becomes obscured by other effects of inoculum size such as altered morphology (Meyrath and Suchanek 1972), the activity of growth-inducing and growth-inhibiting substances of fungal origin (Meyrath 1962), or other control mechanisms (Trinci 1969).

Carbon and nitrogen utilization may be altered by inoculum size in such a way as to affect maximum mycelial yield. These phenomena were studied in Aspergillus oryzae by Meyrath and McIntosh (1963), and McIntosh and Meyrath (1963). It was suggested that the age of mycelium affects

its physiology. A culture generated from a small inoculum requires greater time to achieve the same mycelial mass as a culture generated from a large inoculum. Since equal masses of mycelium generated from different inoculum sizes may be of different age, they may also be different physiologically. This was shown experimentally by lower specific oxygen uptake in small inoculum cultures (specific oxygen uptake is also shown to decrease with culture age), lower economic coefficient (mycelium produced/carbon source consumed) of small inoculum cultures, and decreased assimilation of nitrogen per mass of mycelium produced in small inoculum cultures (Meyrath and McIntosh 1963, McIntosh and Meyrath 1963). It therefore becomes apparent that depletion of carbon source is more rapid in small inoculum cultures, resulting in a lower final yield of mycelium provided that nitrogen or other essential nutrients are not limiting. Fungal growth at the level of hyphal morphogenesis must be considered if the physiological discrepancy between equal mycelial masses raised from inocula of different sizes is to be understood.

It is well established that growth is restricted to hyphal tips, and that hyphae attain a linear rate of elongation. As exponential growth is intuitively expected, it has been suggested that branch initiation corresponds to cell division in unicellular microbes (Trinci 1979). A conceptual difficulty arises, however, in that an

intercalary compartment (a segment of hypha between two branches) cannot continue to contribute to the acceleration of growth without perpetually initiating new branches. Such physical limitations obviate the analogy to growth by binary fission. There is also the phenomenon of hyphal differentiation: hyphal growth occurs with the forward movement of cytoplasm into the elongating tip and the replacement of cytoplasm by vacuoles and lipid deposition in ageing regions of the hypha. Doubling of hyphal length is therefore not contingent upon a doubling of protoplasm (Zalokar 1959).

Elongation rates of hyphae and the development of branching systems have been measured for a number of fungi. For Coprinus disseminatus it was observed that hyphal elongation occurs at a linear rate; however, the elongation rates of main hyphae, primary, and secondary branches were successively less, as was their diameter. Variations in these rates were found to be slight, and branches were reported to occur at regular intervals. The observations were taken from hyphae at colony margins on solid medium (Butler 1961). The more complex system of mycelial strand formation by Merulius lacrymans was also studied; here too, the fundamental nature of hyphal ontogeny was as described above. It was believed that the sequence of branches from the hyphal tip depicted the development of a single branch with time. The growth of hyphae proceded at a linear rate,

with branch formation at regular intervals.

The hypothesis that a fungal growth unit having a constant doubling time exists was proposed by Plomley (1959), based on early colony formation of Chaetomium. By observing the outgrowth of hyphae from individual spores, Trinci (1974) found that the total mycelial length increases exponentially; however, these observations were taken within the time period between germination and the attainment of maximum hyphal extension rate. The initial outgrowth from a spore was shown to be exponential, yet the ensuing hyphal differentiation may modify the rate of growth of a mature colony. Accordingly, a comparison of undifferentiated mycelia and hyphae at colony margins showed that intercalary compartments of the hyphae at colony margins were significantly greater in length. These observations were made with Geotrichum candidum, which differs from the other fungi discussed in that it forms complete septa. Therefore the cytoplasm and nuclei contained between these septa cannot contribute to mycelial growth. The nuclei were observed to be uniformly distributed throughout the mycelium, and each intercalary compartment showed 7±2 nuclei. Spores were formed by fragmentation of the hyphae and consequently contained 1 to 7 nuclei (Fiddy and Trinci 1976B). Clearly then, genetic duplication is restricted to growing regions, and exponential increase of fungal mass is limited to the initial growth of undifferentiated hyphae. In Aspergillus

nidulans, which does not form complete septa, it was demonstrated that the initiation of mitosis was dictated by the volume of cytoplasm per genome (Fiddy and Trinci 1976B).

## III. MATERIALS AND METHODS

### Microorganisms

The *Scytalidium* isolates and wood decay fungi used in this study were obtained from the University of Alberta Mold Herbarium (courtesy of Ms L. Sigler) and from the Northern Forest Research Center, Canadian Forestry Service, Environment Canada, 5320-122 street, Edmonton (courtesy of Dr. Y. Hiratsuka), and are designated by isolate numbers from these sources. These organisms were stored on cereal agar at -20°C, and subcultured every six months. Cultures were also maintained on potato dextrose agar at 4°C as a source of inoculum. Starter cultures were prepared by transferring about 1 $cm^2$ of mycelium to 50 mL of growth medium, homogenizing with a Sorval Omni-mixer at top speed for 10 seconds, and incubating at 27°C with 200 rpm gyratory shaking.

The yeast isolates were obtained from the National Research Council of Canada Prairie Regional Laboratory, Saskatoon. The yeasts were grown on glucose peptone yeast-extract agar and stored at 4°C.

*E. harknessii* teliospores were collected from various locations in Alberta. Spores were dislodged from the aecia onto waxed paper by tapping the galls (Fig. 1). The spores were dried when collected by placing them in open vials in a sealed container with oven-dried silica gel. The spores were later divided into small portions for storage in sealed

Figure 1. The collection of E. harknessii spores from lodgepole pine.

The orange teliospores are dislodged from the gall onto glassine paper by tapping.

microfuge tubes at -20°C. All of the spores used in this study were collected from galls on lodgepole pine.

## Production of S. uredinicola Metabolites

1) Culture procedure

Malt extract broth starter cultures of S. uredinicola 4149 were homogenized after 10 days incubation. A volume of homogenate containing 40 mg wet weight mycelium was used to inoculate four 200 mL volumes of malt extract broth in 500 mL Erlenmeyer flasks. After 10 days incubation, these cultures were combined and used in the same was as the starter cultures to inoculate 80 more flasks. All cultures were incubated at 27°C with 200 rpm gyratory shaking on a New Brunswick Scientific model G11 High Speed Gyrotory Shaker. Wet weight determinations of mycelium in culture homogenates were made by vacuum filtering 10 mL samples onto tared Whatman No. 1 filter paper circles. The vacuum was used to draw air through the filter paper and mycelium for 15 minutes before weighing.

After 10 days growth, the eighty flasks (16 L of culture in total) were harvested by filtration through tared Schleicher and Schuell #520B filter papers, and the weight of mycelium was determined after drying at 100°C for 24 hours.

2) Extraction of crude metabolites

Previous studies had shown that a substance which inhibited the germination of E. harknessii spores could be extracted from the spent medium with chloroform (Fairbairn et al. 1983). Therefore, each 16 L batch of culture filtrate was concentrated to about 500 mL (by rotary evaporation) and extracted two times with 250 mL of chloroform. The chloroform phase was dried over anhydrous sodium sulfate, and evaporated to a viscous brown liquid. About 400 mg of extract was obtained from each 16 L batch of culture.

3) Isolation of the spore germination inhibitor

Sequential fractionation by chromatography with columns of silica gel and Sephadex LH20 was used to isolate the inhibitory component from the crude metabolites. At each step, the inhibitor was detected by the spore germination assay. Gas chromatography (GC), ultraviolet (UV) absorbance scanning, and thin layer chromatography (TLC) were used to characterize each preparation. The procedure by which the inhibitor was purified is described fully in the Results section.

Bioassay Systems

1) Spore germination assays

To detect antifungal activity at each stage of inhibitor purification, the spore germination assay of

Fairbairn et al. (1983) was used. Materials to be tested were dissolved in sterile 20 mM potassium phosphate buffer at pH 6.0. Aliquots (1 mL) in tissue culture wells (Costar 24 well clusters) were dusted with E. harknessii spores, and the extent of germination relative to controls was recorded after 24 hours incubation at room temperature.

To obtain a quantitative measure of inhibitory activity glass slides were prepared with a 2 mm thick layer of 2% agar containing 10 mM potassium phosphate buffer at pH 6.8 and the substance to be tested. The determination of optimal conditions for germination (buffer concentration and pH) for this system is presented in the Results section. Spore suspension (0.05 mL of a 15 mg/mL water suspension) was placed on the agar, and a coverslip was applied. The slides were incubated at room temperature for 24 hours in a moist chamber. Germination was determined by counting the number of spores with a germ tube length exceeding the spore diameter: 100 spores were examined on each of quadruplicate slides, using a light microscope at 400 x magnification.

2) Mycelial growth assays

The activity of the purified inhibitor, maltol, was measured by its ability to inhibit the growth of test organisms in submerged cultures. Sterile stock solutions of maltol (filter sterilized), 4% malt extract broth, and distilled water were combined to give 50 mL of 2% malt

extract broth with the desired concentration of maltol in 125 mL Erlenmeyer flasks. The flasks were inoculated with 1 mL of malt extract broth starter-culture homogenate. Test cultures were incubated at room temperature with 200 rpm gyratory shaking, and the dry weight was determined after substantial growth was observed, usually 48 hours. Duplicate tests and controls were performed.

3) Yeast MIC determinations

Potato dextrose agar (PDA) plates containing maltol at concentrations of 4.0, 2.0, 1.0, and 0.5 mg/mL were prepared. Yeasts were transferred from stock cultures and inoculated onto the plates by a single stab. Minimum inhibitory concentration (MIC) was recorded as the concentration at which no apparent growth occurred after 48 hours incubation at room temperature.

Analytical Procedures

1) Thin layer chromatography

Silica gel thin layer chromatograms (Merck 60 $F_{254}$, 20 cm x 20 cm, plastic backed, 0.2 mm layer thickness) were developed with chloroform:methanol (94:6, v:v). The chromatograms were visualized under shortwave and longwave UV light, and with iodine vapor.

2) Gas chromatography

For gas chromatographic analyses, samples were dissolved in chloroform, and 3 µL of the chloroform solution was manually injected. A Hewlett Packard model 5730 gas chromatograph was used with a 0.25 mm by 30 m SE-54 fused silica capillary column (J and W Scientific) and a flame ionization detector. The carrier gas was He at a linear velocity of 27 cm/second at 100°C with $N_2$ at 20 mL/minute as a make-up gas. The temperature program used was 90°C to 250°C at 8°C per minute. The chromatogram, retention times, and peak areas were recorded by a Hewlett Packard model 58802A integrator operating at a chart speed of 0.5 cm/minute.

3) Ultraviolet absorbance scanning

UV scans were obtained using a Pye Unicam SP8-100 spectrophotometer. All scans were from methanol solutions (unless stated otherwise), recorded at 1 nm/second scan speed and 10 seconds/cm chart recorded speed. Quartz cuvettes with a 1 cm light path were used.

Growth Curves

1) Inoculation of cultures grown on synthetic medium

Inoculum cultures were prepared from starter cultures as described previously, except that synthetic medium was used throughout. Inoculum cultures were pooled and homogenized after 10 day incubation, and the mycelium was

separated from the medium by centrifugation. The mycelium was resuspended in fresh medium to the inoculum density desired for each experiment. Cultures used for growth experiments were inoculated with 10 mL of suspension to give final culture volumes of 200 mL in 500 mL, foam plugged Erlenmeyer flasks.

2) Dry weight determination

Cultures were harvested by vacuum filtration through tared Whatman #1, 7 cm filter paper circles. Culture filtrate volumes were recorded, and the filtered mycelium was washed with water and dried for 24 hours at 100°C before weighing.

3) Determination of maltol concentration in growth media

The culture filtrates were adjusted to pH 3.5 with N HCl or NaOH, and extracted with an equal volume of chloroform. The chloroform phase was dried over anhydrous sodium sulfate, reduced to 1.0 mL, and a 3 µL sample was analyzed by gas chromatography. The concentration of maltol in the medium was estimated from a standard curve.

4) Glucose determination

Glucose concentration in synthetic medium cultures was determined using Sigma's Glucose diagnostic kit, following the method of Keston (1956). Samples of spent medium (0.75

mL) were diluted to 50 mL with distilled water. for analysis, 0.05 mL of the diluted sample was combined with 5.0 mL of enzyme/color reagent and incubated at room temperature for 45 minutes. Glucose concentration was determined by the absorbance at 450 nm.

5) Total carbohydrate

Total carbohydrate assays of synthetic medium cultures were conducted by the o-toluidine method of Dubois et al. (1962). Duplicate 0.5 mL samples of culture filtrate were combined with 1 mL of 5% phenol followed by 4 mL of 98% sulfuric acid. Absorbance at 490 nm was recorded after 30 minutes at room temperature.

6) Ammonium determination

Ammonium concentration in synthetic medium cultures was determined following the method of Weatherburn (1967), which uses the Berthelot color reaction. The standard protocol was followed, using 20 µL samples of culture filtrate, and an incubation time of 23 minutes at room temperature.

7) Nitrate determination

Nitrate concentration in synthetic medium cultures was determined by the method of Rebelein (1967, described by Amerine and Ough, 1980).

Measurement of Hyphal Growth

S. uredinicola spores were transferred with a bacteriological loop from mycelium on PDA plates to layers of PDA on microscope slides. A coverslip was applied and phase contrast light micrographs were taken of hyphal outgrowth at 1 hour intervals. The slides were incubated in moist chambers at room temperature between photographs.

An alternative method was used to examine hyphal growth at the margins of mature colonies. Glass slides were placed in 9 cm petri dishes and overlaid with 10 mL of PDA. colonies of S. uredinicola 4149 were grown over the slides from spore inoculum. The glass slides with the agar overlaying them were excised from the plates when the colony diameter approached 2 cm. Coverslips were applied over the colonies, and time-lapse photomicrographs were taken. Measurements were taken from photographs printed at 380 x magnification.

In Vivo Testing of Maltol as a Fungistatic Agent in Pine Seedlings

1) Growth of pine seedlings

Seeds collected from open pollinated lodgepole pine near Grand Prairie, Alberta (courtesy of Dr. P. Blenis, Department of Plant Science, University of Alberta) were planted in peat moss with biweekly application of 20-20-20 fertilizer. Ferdinand Rootrainers (Spencer-Lemaire

Industries Ltd., Edmonton, Alberta) were used. Each tray had 96 cavities, and 4 seeds were planted in each cavity. The trays were maintained at 20°C constant temperature under natural light (supplemented by 400 W high pressure sodium lamps for 12 hours on cloudy days or when the natural photoperiod was less than 12 hours).

2) Application of maltol

Aqueous maltol (2 mL) was pipetted onto the soil of each cavity 2 days prior to inoculation of the seedlings with E. harknessii spores. The maltol concentrations used were 10, 1.0, 0.1, and 0.01 mg/mL. Control groups received 2 mL of distilled water.

3) Inoculation with E. harknessii spores

E. harknessii spores were suspended in distilled water to 3 mg/mL, and applied to the pine seedlings using a chromatography sprayer. Each tray of seedlings received 20 mL of suspension. In all cases, spore viability of the suspensions was greater than 95% when assayed by the spore germination assay described previously. Inoculation took place between 56 and 60 days after planting. After applying the spore suspension, the plants were contained in a humidified chamber at 18°C in the dark for 48 hours to facilitate germination (P. Blenis personal communication 1985).

## IV. RESULTS

### Culture Conditions and Inhibitor Production

The effect of inoculum quantity on culture growth rate and mycelial yield was investigated by using different volumes of a single inoculum preparation. Four growth curves were generated as shown in Fig. 2. The results of this experiment indicated that both the maximum yield and growth rate increased with inoculum size. The mass of inoculum selected for inhibitor production cultures was 0.04 grams wet weight, such that the cultures would be entering stationary phase after 10 days incubation. The mean mycelial yield ± standard error (SE) between the 16-L batches used for inhibitor production was 4.1 ± 0.5 g/L (dry weight).

After the sporostatic compound had been identified as maltol, a growth curve experiment was performed in which medium maltol concentration was followed. Medium pH was also monitored because the early rate of acid production provided a means of predicting culture development. The results of this experiment are shown in Fig. 3. It is evident that the presence of maltol in the growth medium was transient, and it was correlated with the growth curve such that the peak concentration of maltol occurred early in the stationary phase. In the experiment shown, the inoculum size was less than that used for inhibitor production cultures as described above.

Figure 2. The effect of inoculum size on culture growth rate and mycelial yield.

Erlenmeyer flasks (500 mL) containing 200 mL malt extract broth were inoculated with 44 mg (●), 30 mg (●), 15 mg (■), or 3.7 mg (▲) wet weight of mycelium, and incubated at 27°C with 200 rpm gyratory shaking. The inoculum was homogenized mycelium from a 10 day old culture grown under the same conditions, containing about 7 mg/mL wet weight mycelium.

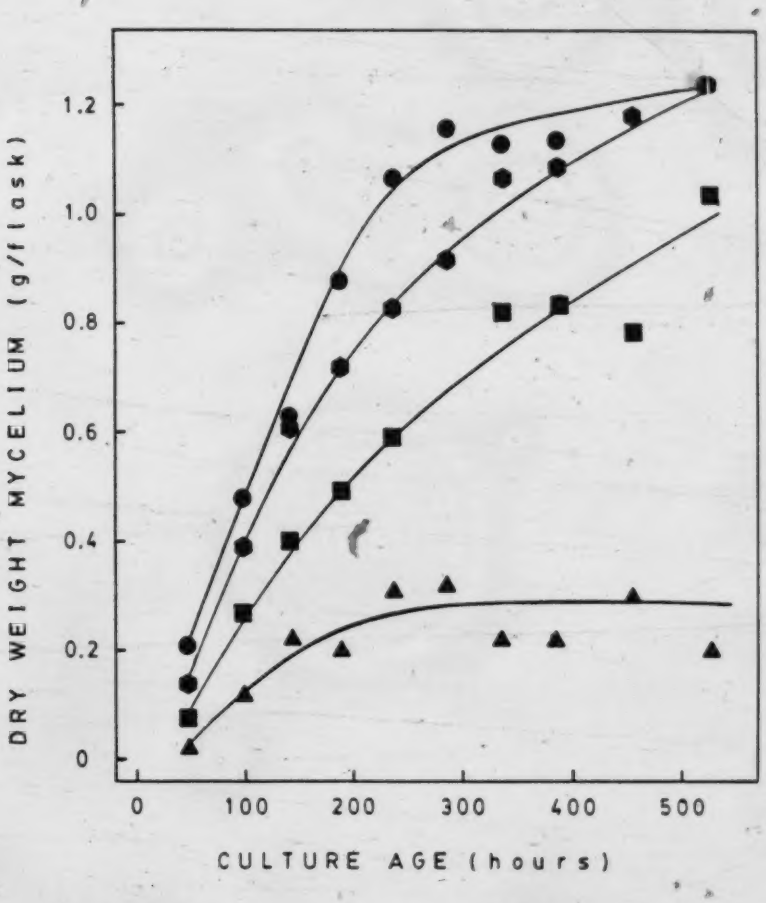
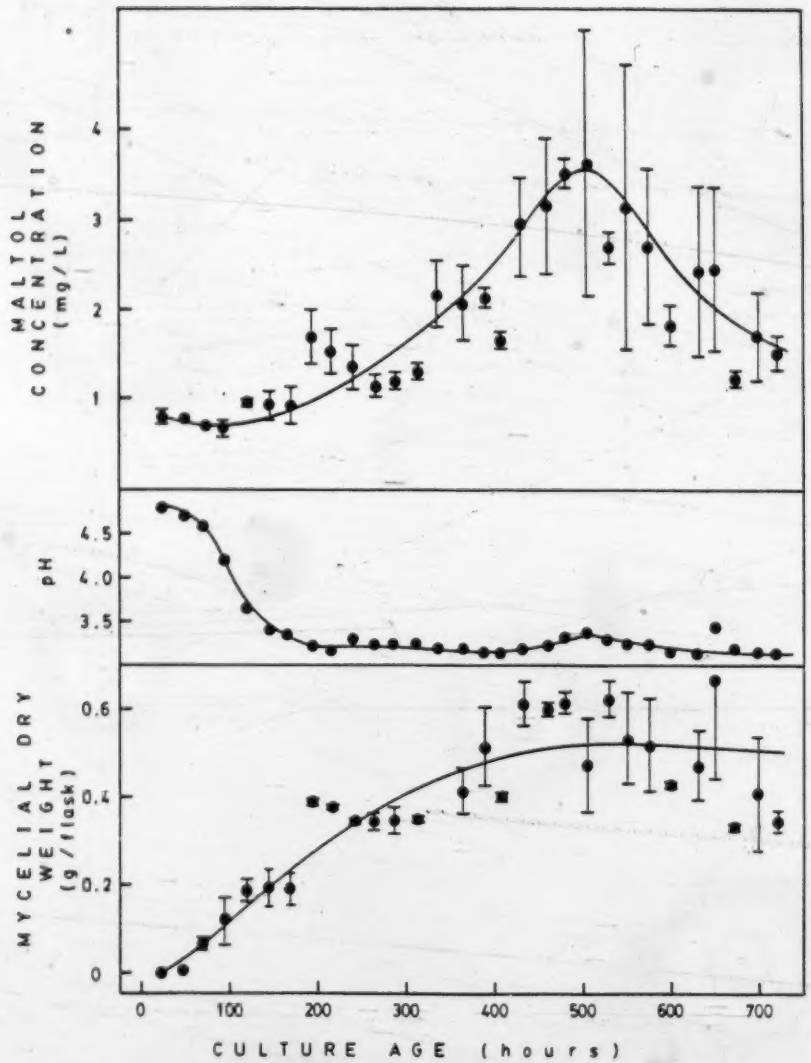

DRY WEIGHT MYCELIUM (g/flask)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
CULTURE AGE (hours)

Figure 3. The relation of maltol concentration, medium pH, and mycelial yield with time in submerged cultures of S. uredinicola 4149.

Erlenmeyer flasks (500 mL) containing 200 mL of 2% malt extract broth were inoculated with 4 mL of S. uredinicola 4149 culture homogenate. The flasks were incubated at 27°C with 200 rpm gyratory shaking. Duplicate flasks were harvested at 1 day intervals for 30 days, and analyzed for maltol content, mycelial dry weight, and pH. Error bars represent standard error of the mean.

The inoculum mass was 40 mg wet weight mycelium per flask. Presumably variation in the inoculum preparation between experiments accounts for the different mycelial yield here as compared to Fig. 2.


MALTOL CONCENTRATION (mg/L)
1
2
3
4
pH
4.5
4.0
3.5
MYCELIAL DRY WEIGHT (g/flask)
0
0.2
0.4
0.6
0
100
200
300
400
500
600
700
CULTURE AGE (hours)

Consequently, slower growth, lower mycelial yield, and a delay in the time required to reach stationary phase were observed.

## Inhibitor Purification and Identification

1) Mobility of Inhibitory Substances on TLC

The chloroform extract from 8 L of S. uredinicola culture was applied to a preparative TLC (Analtech Inc., Redi/Plate Silica gel GF, 0.25 mm layer thickness), and developed with chloroform:methanol (94:6, v:v). This was done to determined if all of the inhibitory substances were mobile over silica, and therefore whether silica gel column chromatography would be appropriate for purification. The thin layer was then divided into bands of 0.1 $R_f$ units, and each was extracted with 10 mL of distilled water. The silica was sedimented from the aqueous solution; 6 mL of each supernatant was combined with 6 mL of buffered, molten agar (4%), and used for quadruplicate germination assays. The results of this experiment, shown graphically in Fig. 4, indicate that the inhibitory activity is located between $R_f$ 0.3 and $R_f$ 0.7.

The substances recovered from $R_f$ 0.2 to $R_f$ 0.3 caused abnormal germ tube ontogeny, and multiple germ tube formation from single spores; as many as six germ tubes from individual spores were observed. Photomicrographs of the anomolous germination phenomena are shown in Fig. 5. Thin layer chromatography did not provide sufficient purification of the inhibitory substances for chemical characterization.

Figure 4. The distribution of sporostatic activity on a preparative thin layer chromatogram.

S. uredinicola 4149 was grown in 500 mL Erlenmeyer flaks with 200 mL malt extract broth. The cultures were inoculated by the standardized procedure, and incubated at 27°C for 10 days. The culture medium (8 L) was then extracted with chloroform, and the extracted materials were chromatographed over silica gel with chloroform: methanol (94:6, v:v). Spore germination in the presence of materials recovered from each $R_f$ band was determined by the slide method. Bars represent standard error of the mean.

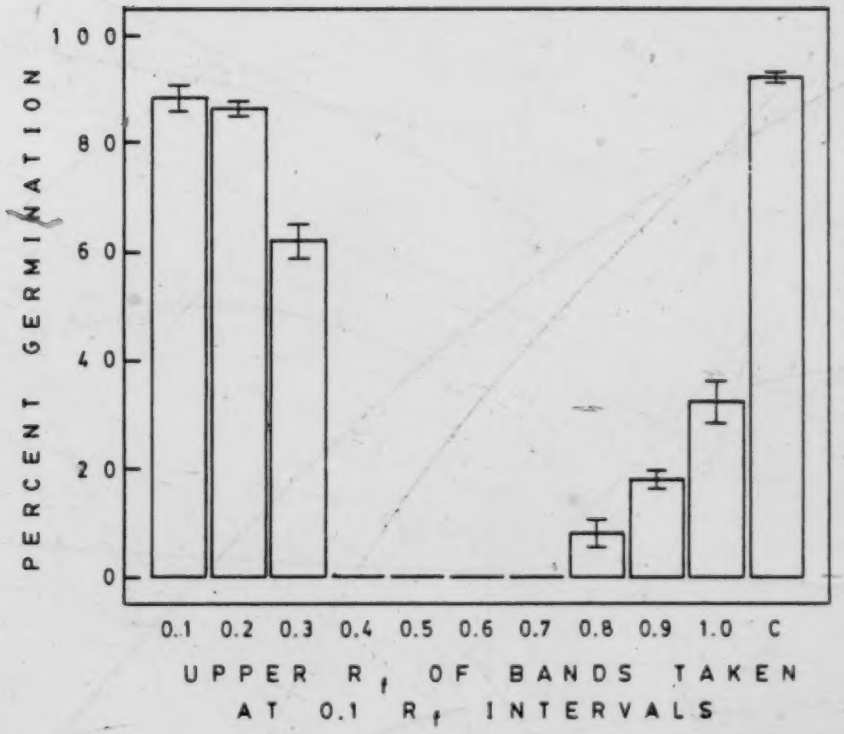
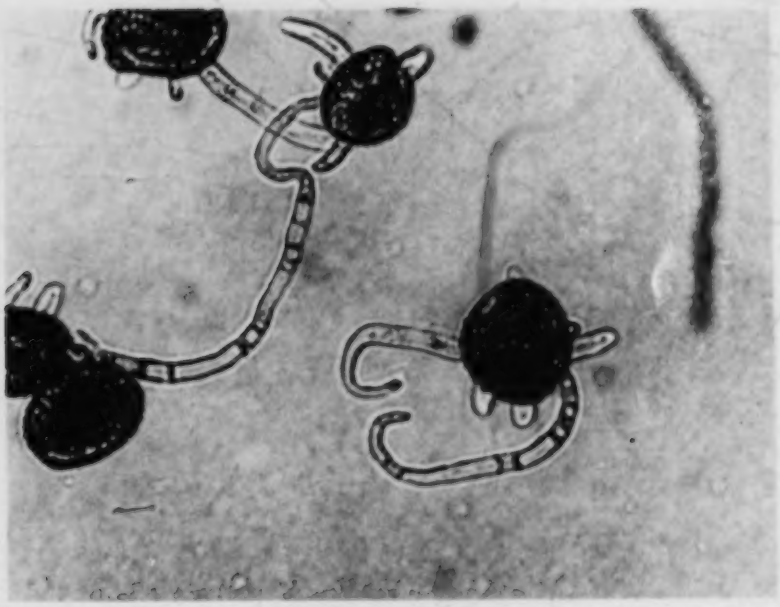

PERCENT GERMINATION
100
80
60
40
20
0
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 C
UPPER $R_f$ OF BANDS TAKEN
AT 0.1 $R_f$ INTERVALS

Figure 5. Photomicrographs of abnormal spore germination caused by S. uredinicola metabolites recovered from preparative thin layer chromatography.

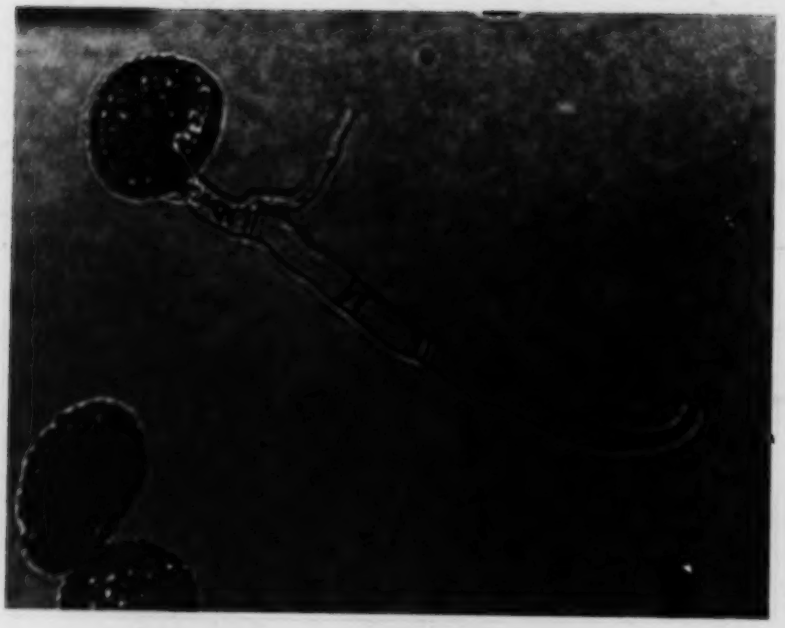
The materials recovered from $R_f$ 0.2 to 0.3 were observed to cause multiple germ tube formation by E. harknessii spores. Photomicrographs A (480 x magnification) and B (660 x magnification) show the effect of the S. uredinicola metabolites. Photomicrograph C (670 x magnification) shows a spore germinating normally under control conditions.

A



B

C



2) Isolation of the Inhibitory Metabolite by Column Chromatography

A sequence of four chromatographic columns was used to isolate the inhibitory substance from culture extracts. Two large columns, the first of silica gel and the second of Sephadex LH20, were used for a coarse fractionation of the extract to prevent overloading of the subsequent columns. The inhibitory preparation obtained from the first two columns was purified by repeating the process, using columns designed for maximum resolution. The results of the purification scheme are described below; the columns are numbered in the sequence they were used.

Column I:

The initial fractionation of crude extracts was carried out by adsorption column chromatography over silicic acid (Mallinkrodt CC-7), in an adaptation of the method of Hirsch and Aherns (1958). Forty grams of oven-dried silica was packed dry into a 2.5 cm diameter glass column with a filter paper disc above and below the bed. The bed was settled by tapping the column, and subjected to a dehydrating acetone wash before equilibration with chloroform. The resulting bed height was 20 cm. The crude extract, about 400 mg, was applied to the column and eluted with chloroform. The fractions collected were approximately 15 mL.

Bioassay of each fraction was carried out by evaporating a 1 mL sample and dissolving the residue in 2 mL

of buffer for duplicate germination assays by the well method. Thin layer chromatographic analysis was performed by spotting 5 µL of each fraction at 0.5 cm intervals along the origin of the TLC plates. Fig. 6 shows the results of TLC analysis: in the experiment shown, fractions 19 through 23 caused inhibition of spore germination. In fraction 17 to 24, only one spot was visible under shortwave UV light and with iodine, and it occured at $R_f$ 0.4. Gas chromatographic analysis of fraction 21, presented in Fig. 7, indicated that at least 7 compounds were present in substantial amounts. Fractions 12 through 30 were pooled, yielding about 50 mg of red-brown oil after evaporation of the solvent.

Column II:

A column of Sephadex LH20 (40 cm by 2.5 cm) in methanol was prepared. The inhibitor-containing material obtained from column I was applied and eluted from this column with methanol; fractions of approximately 4 mL were collected. Fig. 8 shows four distinctively colored and well separated bands that were visible during the run. For each fraction, the residue from a 50 µL sample was dissolved in 1 mL of buffer and used to conduct a spore germination assay by the well method. Sporostatic activity was detected in fractions 56 through 58. These fractions were pooled, and GC analysis of the pool detected the presence of only two compounds. However, subsequent column procedures indicated that

Figure 6. Thin layer chromatographic analysis of crude extract fractions obtained by silica gel column chromatography.

The chloroform extract from 16 L of malt extract broth culture of S. uredinicola 4149 was eluted from a column of silica gel (2.5 cm diameter, 20 cm bed height) with chloroform. Each fraction (15 mL) was analyzed by thin layer chromatography using a chloroform: methanol (94:6, v:v) solvent system. The chromatogram was visualized with iodine vapor and photographed; the drawing is a composite of two TLCs (fractions 1-20 and fractions 21-30) using $R_f$ values determined from the photographs.

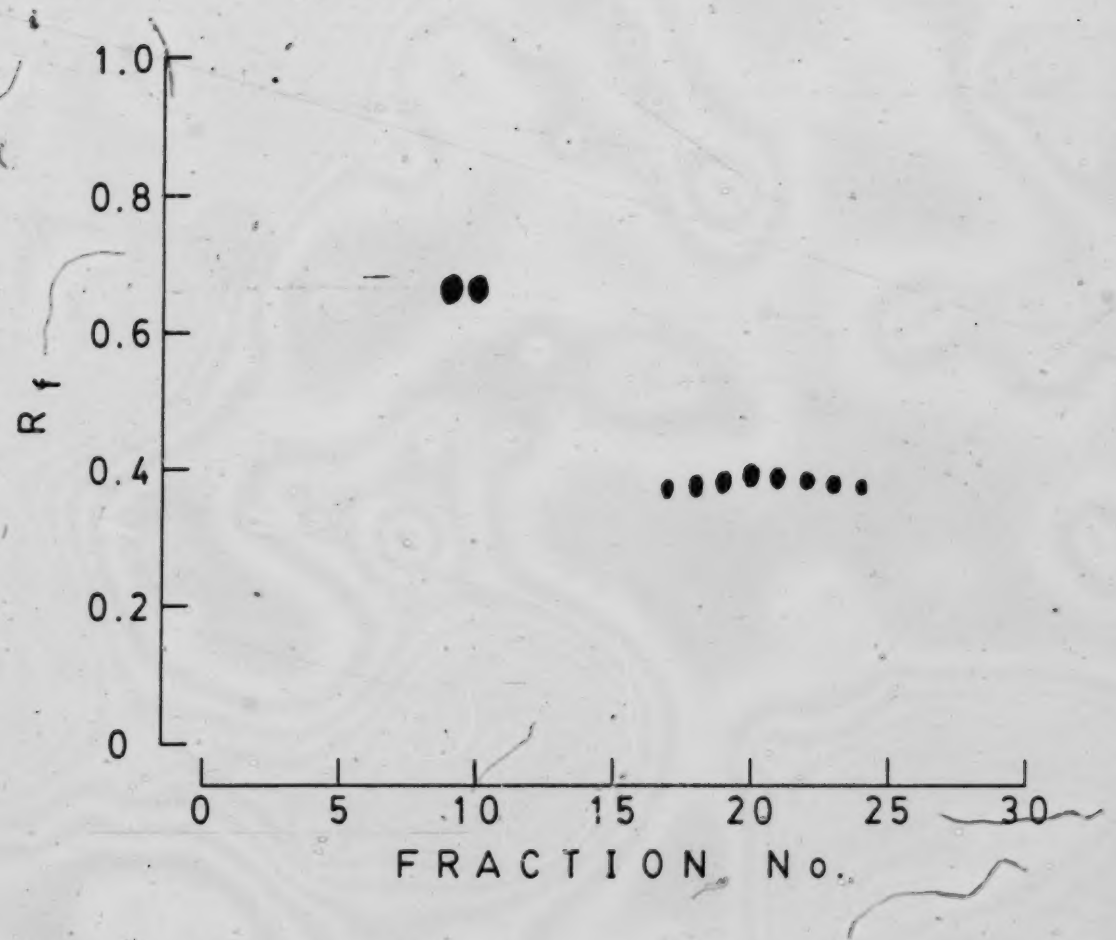

1.0
0.8
0.6
0.4
0.2
0
$R_f$
0
5
10
15
20
25
30
FRACTION No.

Figure 7. Gas chromatographic analysis of fraction 21 from chromatography column I of the inhibitor purification procedure.

The chloroform extract from 16 L of malt extract broth culture of S. uredinicola 4149 was eluted from a column of silica gel (2.5 cm diameter, 20 cm bed height) with chloroform. Fraction 21, which contained a sporostatic component, was analyzed by gas chromatography. The temperature program used was 90°C to 250°C at 8°C per minute. Using a flame ionization detector, the fraction was found to have at least 7 compounds present.

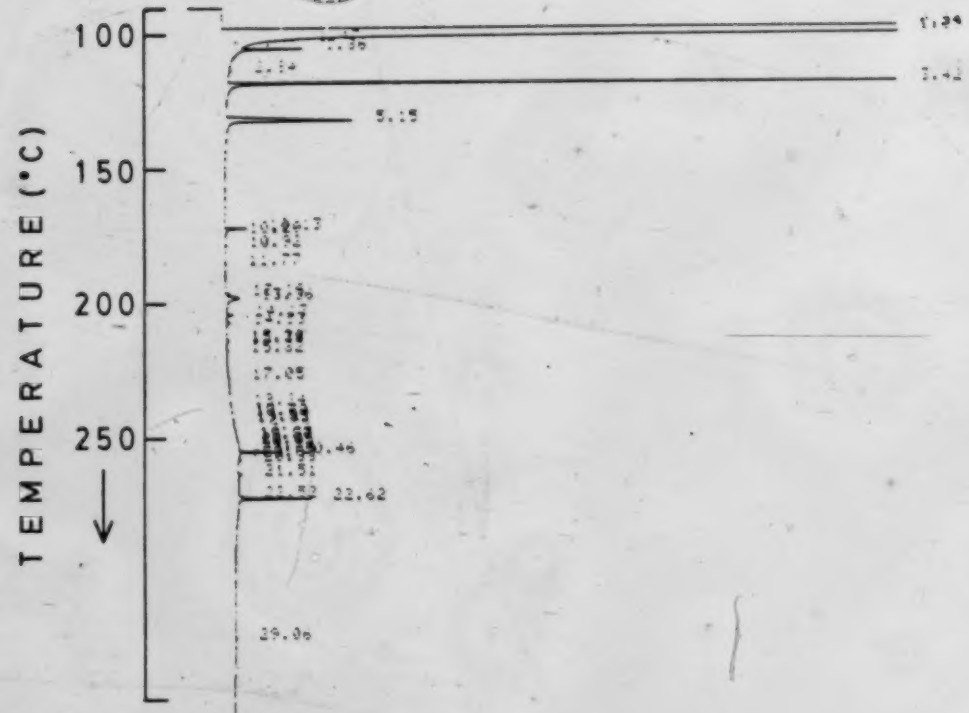
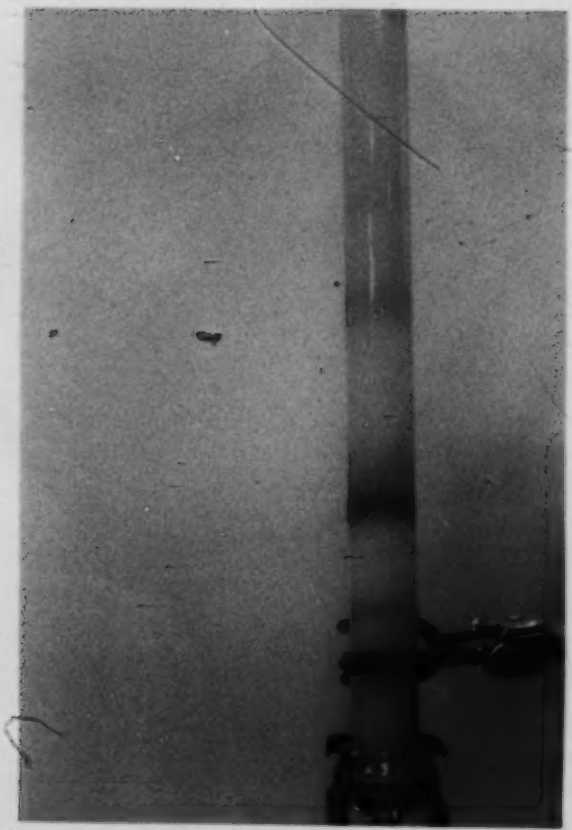

TEMPERATURE (°C)
100
150
200
250
22.62
29.06

Figure 8. Photograph of chromatography column II in the inhibitor purification procedure.

Fractions 12 to 30 from silica gel chromatography of S. uredinicola culture extract, as shown in Fig. 6, were pooled. The materials in this pool were fractionated by column chromatography using sephadex LH20. The column diameter was 2.5 cm, the bed height 40 cm, and the eluant was methanol. The photograph shows the progression of distinctively colored bands that occurred; sporostatic activity was associated with the large, reddish band second from the bottom.



other contaminating materials were present. Attempts to crystallize the inhibitor at this stage were unsuccessful.

Column III:

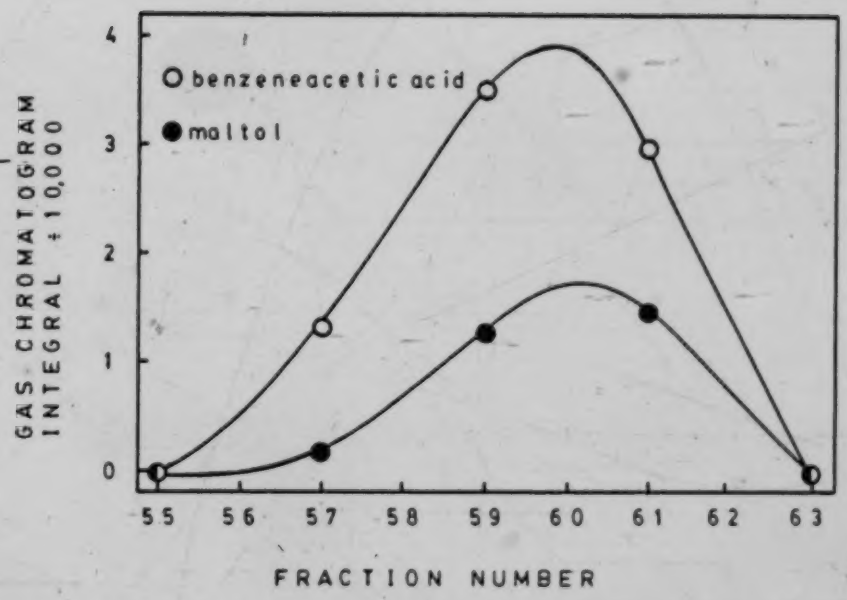
To construct column III, 100-mesh silicic acid (Mallinkrodt) was oven-dried (105°C overnight) and packed dry into a 0.5 cm inside diameter glass tube to a height of 62 cm. The bed was settled by tapping with a glass rod. Filter paper discs were placed above and below the bed; the column outlet consisted of a stainless steel needle held in place by a sleeve of silicone tubing. The column was washed with acetone prior to equilibration with chloroform. The inhibitor-containing preparation obtained from column II was applied and eluted from column III with chloroform, and 2.5 mL fractions were collected. The chloroform eluant was under 20 psi (gauge) entering the column to provide an adequate flow rate. A visibly large portion of the sample was retained at the top of the column. Spore germination assays located the inhibitory activity in fractions 58 through 61; GC analysis of these fractions detected only two compounds, as in the preparation obtained from column II. These substances were later identified as benzeneacetic acid and maltol, and their elution profiles from column III are shown in Fig. 9.

Column IV:

A column of Sephadex LH20 (120 cm by 0.8 cm) in methanol was prepared. The inhibitor-containing preparation

Figure 9. The elution profiles of maltol and benzeneacetic acid from column III of the inhibitor purification procedure.

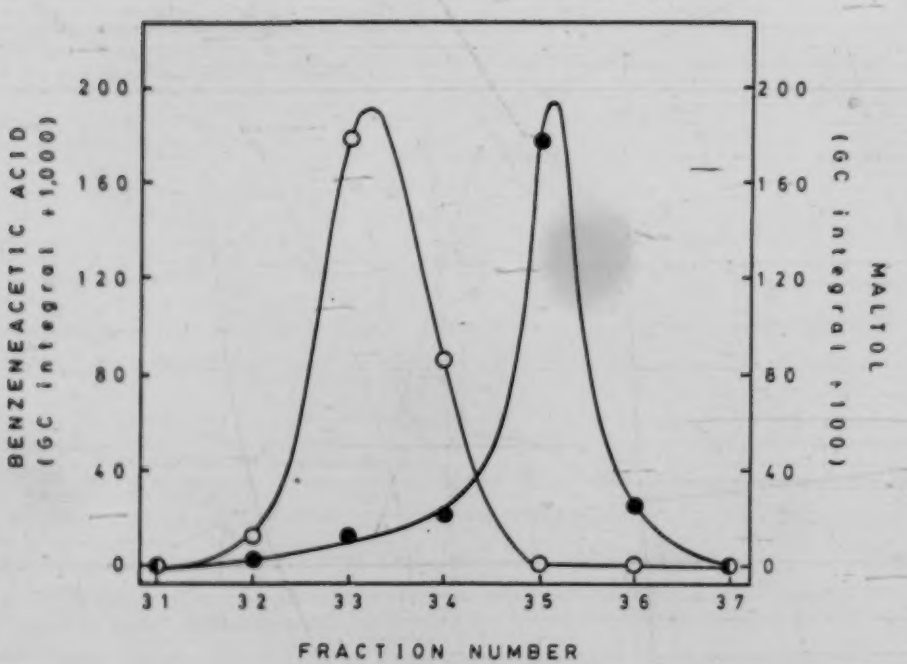
The sporostatic fraction of the S. uredinicola culture extract obtained from chromatography column II in the purification procedure was applied to column III. The column was packed with 100-mesh silica gel to a height of 62 cm, and the bed diameter was 0.5 cm. The metabolites were eluted with chloroform, and 2.5 mL fractions were collected. The fractions possessing sporostatic activity were analyzed by gas chromatography. Only two compounds were detected. The chromatogram integrals were used to plot elution profiles because the compounds had not been identified, and therefore standards were not available to convert the integral units to concentration. The two, unresolved compounds were later identified as maltol and benzeneacetic acid.


GAS CHROMATOGRAM
INTEGRAL ÷10,000
benzeneacetic acid
maltol
0
1
2
3
4
55
56
57
58
59
60
61
62
63
FRACTION NUMBER

obtained from column III was applied and eluted from this column with methanol, and 1.5 mL fractions were collected. The fractions containing the sporostatic substance were found by the spore germination assay; GC analysis of these fractions detected only one compound which was later identified as maltol. The separation of benzeneacetic acid and maltol, which was not achieved by chromatography over silica, is shown by the elution profiles in Fig. 10. Another band was observed to elute from the column with a greater retention volume than benzeneacetic acid; however, it was not detectable by the GC method, nor was it associated with sporostatic activity. A UV absorbance scan and the inhibition of spore germination caused by the inhibitory preparation was recorded. After identification of the inhibitor as maltol, this information was used to ensure that the inhibitory activity was accounted for by the amount of maltol present, by comparison with an authentic sample.

Figure 10. The elution profiles of maltol and benzeneacetic acid from column IV of the inhibitor purification procedure.

The sporostatic fractions obtained from the third column of the inhibitor purification procedure contained two gas chromatography-detectable compounds. The substances in the pooled fractions from the third column were eluted from a Sephadex LH20 column (0.8 cm diameter and 120 cm bed height) with methanol as the eluant. The two gas chromatography-detectable compounds were separated by this procedure. The chromatogram integrals were used to plot elution profiles because the compounds had not been identified, and therefore standards were not available to convert the integral units to concentration. The two compounds were subsequently identified as maltol (●) and benzeneacetic acid (○); sporostatic activity was associated with the maltol-containing fractions only.


BENZENEACETIC ACID
(GC integral ×1,000)
MALTOL
(GC integral ×100)
200
160
120
80
40
0
31
32
33
34
35
36
37
FRACTION NUMBER

## Identification of the Inhibitory Metabolite From S. uredinicola

Gas chromatography-mass spectral analysis with computer library matching identified the inhibitory compound as maltol, or 3-hydroxy-2-methyl-4H-pyran-4-one. The chemical structure is shown in Fig. 11. The mass spectra of the purified inhibitor and its computer library match are shown in Fig. 12; the UV scans of the purified inhibitor and an authentic sample of maltol (Aldrich Chemical Company) are presented in Fig. 13. Gas chromatograms of the purified inhibitor, authentic maltol, and a mixture of the two each showed a single peak.

Figure 11. The structural formula of maltol.

The inhibitory metabolite isolated from S. uredinicola was identified as maltol (3-hydroxy-2-methyl-4H-pyran-4-one).

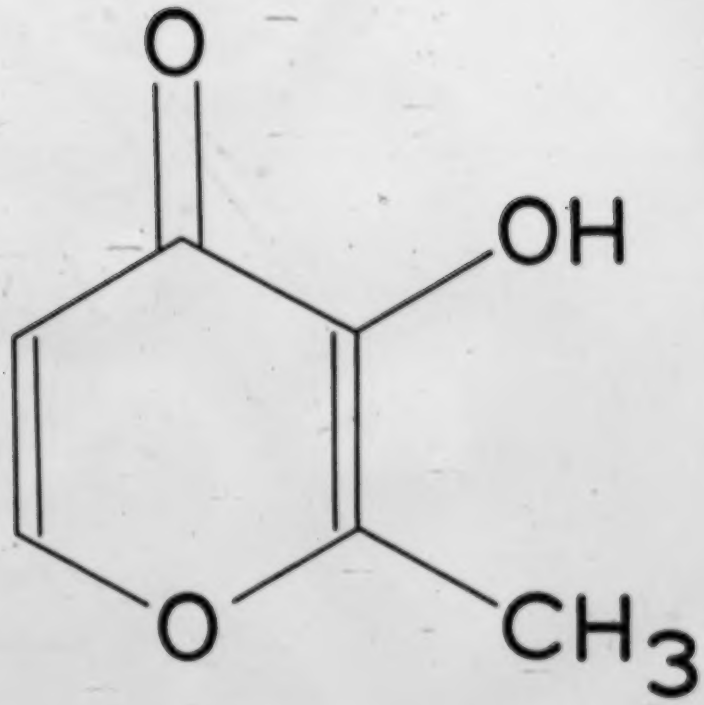

O
OH
O
CH3

Figure 12. Mass spectra of the purified, sporostatic metabolite from S. uredinicola and its computer library match, maltol.

Gas chromatography-mass spectral analysis was obtained for the sporostatic metabolite isolated from S. uredinicola (A); its computer library match was maltol (B).

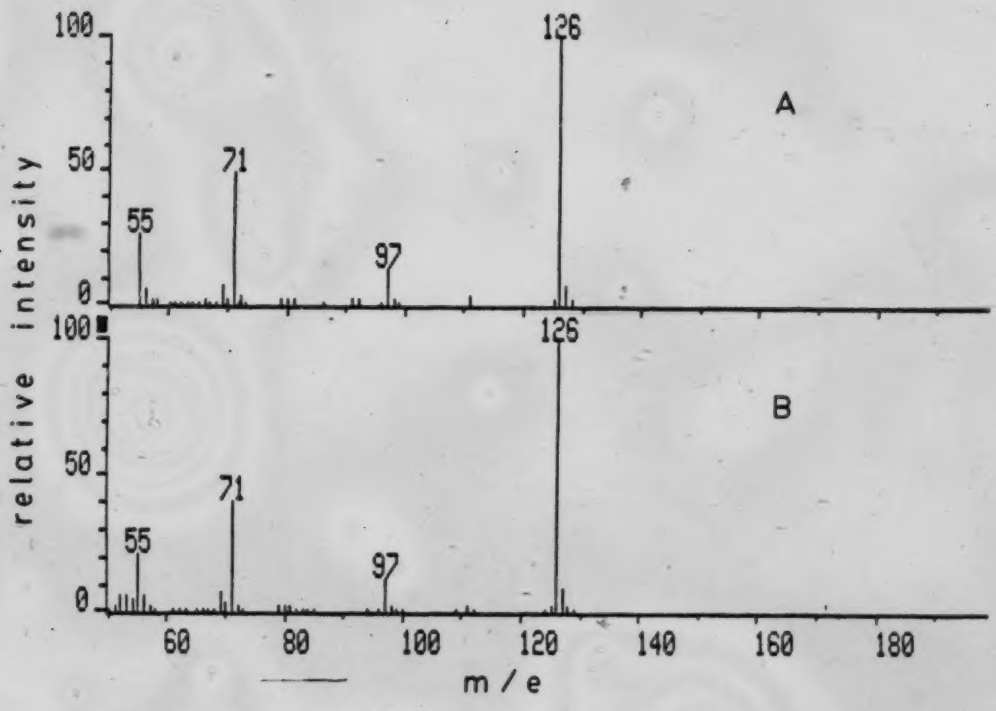
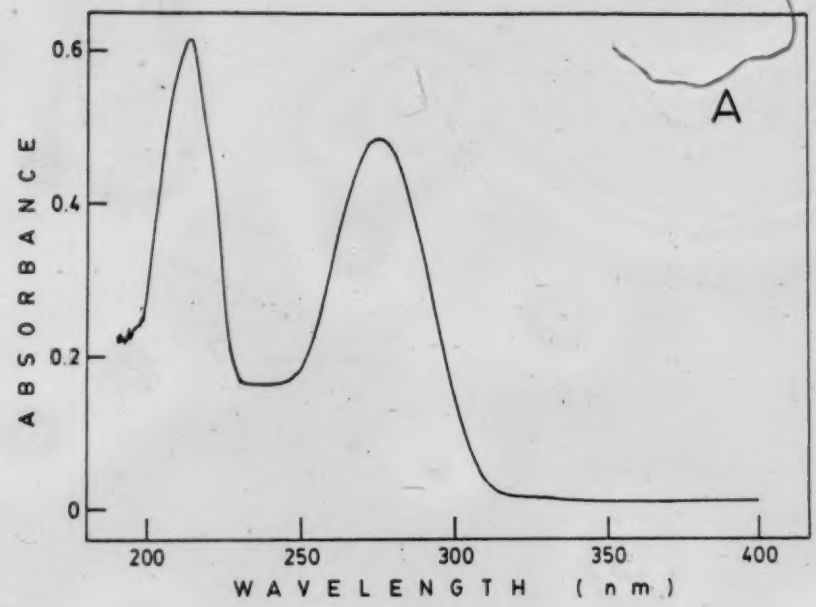
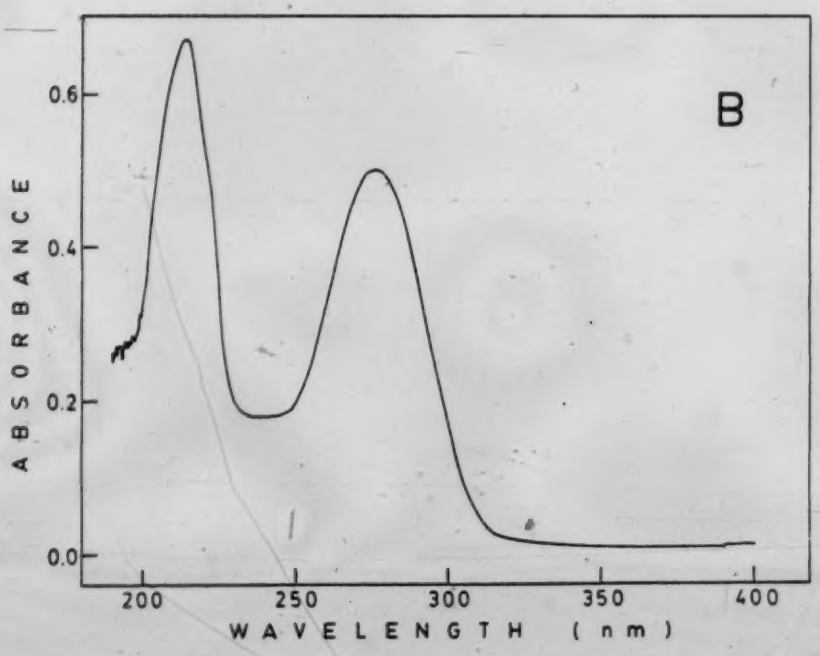

100
50
0
relative intensity
126
71
55
97
A
B
60
80
100
120
140
160
180
m / e

Figure 13. UV absorbance scans of the purified, sporostatic metabolite of S. uredinicola and an authentic sample of maltol.

The sporostatic metabolite isolated from S. uredinicola was scanned for UV absorbance (A), and compared to an authentic sample of maltol (B). Both samples were in methanol; the authentic maltol sample was at a concentration of 1.0 mg/mL.


A
ABSORBANCE
0.6
0.4
0.2
0
200
250
300
350
400
WAVELENGTH (nm)


B
ABSORBANCE
0.6
0.4
0.2
0.0
200
250
300
350
400
WAVELENGTH (nm)

Other Metabolites of S. uredinicola

A compound occurring at $R_f$ 0.63 on the analytical chromatograms was frequently associated with inhibition of spore germination observed by the well method. The compound, obtained by silica gel column chromatography (as shown in the TLC analysis of column fraction 10 in Fig. 6), was purified by precipitation from either chloroform/hexanes or methanol/water to yield a white, friable material. GC-mass spectral analysis provided the tentative formula $C_{11}H_{10}O_4$, but a computer library match was not found. The mass spectrum is shown in Fig. 14, and a UV absorbance scan in Fig. 15. The purified compound was sparingly soluble in water, and at saturation was without inhibitory properties; therefore, the chemical identity was not pursued.

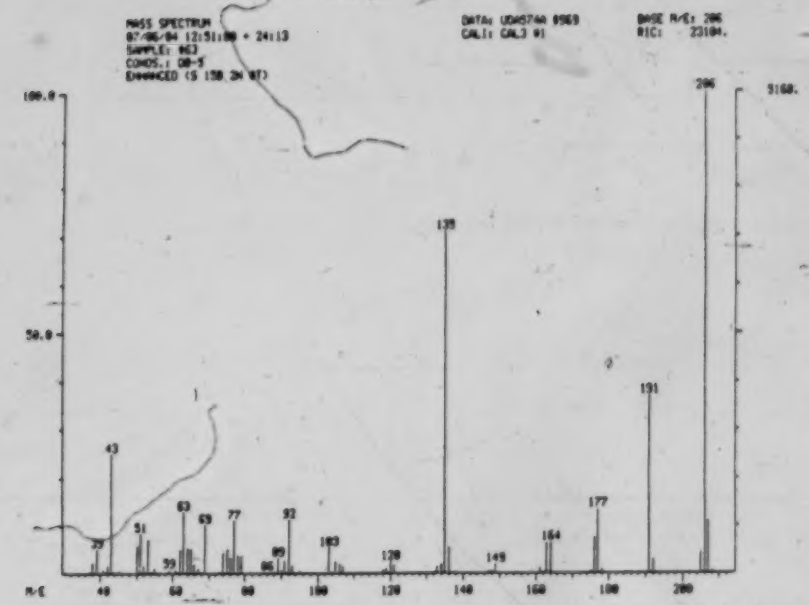
The well-method bioassay of column fractions and materials recovered from chromatograms was reexamined using higher magnification: droplets of a water-immiscible, oil-like substance which co-chromatographs with the $C_{11}H_{10}O_4$ compound were observed at the surface of the wells, and readily adsorbed to the spores upon contact. When present in sufficient quantity to coat the spore surface, this material caused complete loss of spore color, and in some instances lysis of the spores resulted. Germination often preceded these effects. Germination was not inhibited by these preparations in the slide method, nor were these

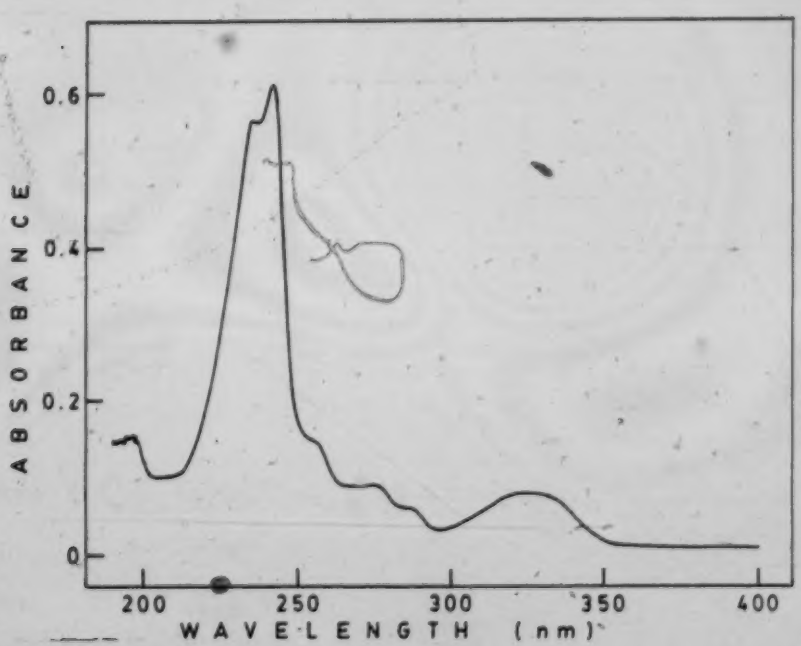
Figure 14. The mass spectrum of an unidentified S. uredinicola metabolite.

One of the most abundant metabolites in the extracts of S. uredinicola culture was isolated by precipitation from chloroform/hexanes. The purified compound was not active against E. harknessii spore germination. A tentative formula ($C_{11}H_{10}O_4$) was assigned by GC-mass spectral analysis, but a computer library match was not found.

Figure 15. The UV spectrum of an unidentified S. uredinicola metabolite.

One of the most abundant metabolites in the extracts of S. uredinicola culture was isolated by precipitation from chloroform/hexanes. The purified compound was not active against E. harknessii spore germination. An aqueous solution of the compound was scanned for UV absorbance.


MASS SPECTRUM
100.0
50.0
206
3160.
135
191
177
43
63
69
77
92
164
M/E
40
60
80
100
120
140
160
180
200

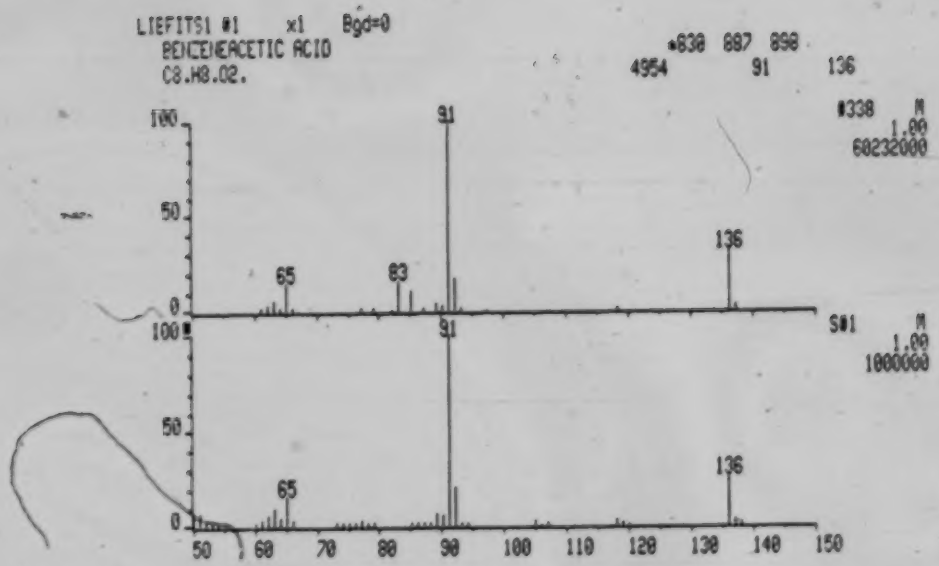

ABSORBANCE
0.6
0.4
0.2
0
200
250
300
350
400
WAVE-LENGTH (nm)

substances inhibitory against the hyphal growth of other fungi; therefore, the effect was regarded as an artefact peculiar to the assay system.

The compound which migrated closely with maltol in the inhibitor purification procedure was obtained in pure form from column IV, and it was identified as benzeneacetic acid by GC-mass spectral analysis with computer library matching (Fig. 16). Benzeneacetic acid (Sigma) at 2.4 mM had no effect on E. harknessii spore germination assayed by the slide method.

Figure 16. Mass spectra of a purified, S. uredinicola metabolite and its computer library match, benzeneacetic acid.

A metabolite of S. uredinicola which was closely associated with inhibitory activity in the inhibitor purification procedure was identified as benzeneacetic acid by gas chromatography-mass spectral analysis. Benzeneacetic acid was not found to be active against E. harknessii spore germination.


LIBFITS1 #1 x1 Bgd=0
BENZENEACETIC ACID
C8.H8.O2.
4954
#838 887 898
91 136
#338 M
1.00
60232000
S#1 M
1.00
1000000
100
50
0
91
65
83
136
50 60 70 80 90 100 110 120 130 140 150

S. uredinicola Growth Kinetics, Carbon and Nitrogen Utilization, and Maltol Production.

1) Effect of inoculum size on growth, pH and maltol production in malt extract broth.

Different volumes of a single inoculum preparation were used to generate four growth curves; growth, medium maltol concentration and pH were determined at 2 day intervals. The results of this experiment are shown in Fig. 17. The time course of maltol production observed in the two curves generated from the largest inoculum sizes are corroborated by the more detailed study presented in Fig. 3. These data establish that there is a connection between the peak maltol concentration and the metabolic phase of the culture, and it is evident that the time course of maltol production is determined by the inoculum size. Also, different inoculum sizes caused changes in mycelial morphology which may have affected metabolite production. In general, maltol production appeared to be delayed and depressed as inoculum size was reduced.

Medium pH was monitored as a measure of metabolic activity. The rate of acid production in the early growth phase reflected the culture growth rate, and served as a predictor of the time of maximum maltol concentration. This parameter was of greater significance when the culture volume was scaled up to 10 L fermentation vessels, and sampling for mycelial mass was subject to greater error.

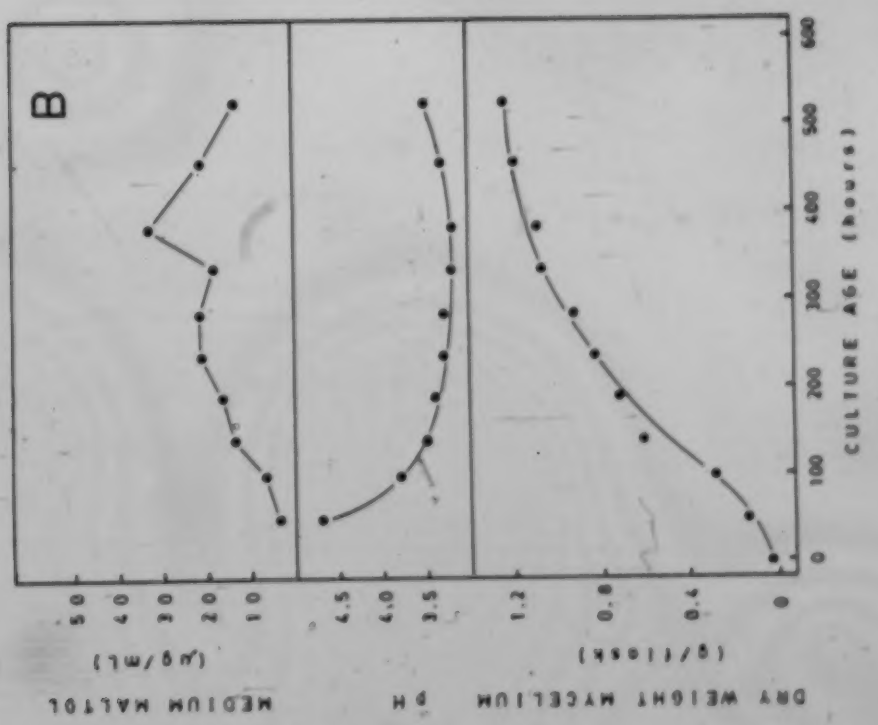
Large scale culture of S. uredinicola was undertaken to demonstrate the feasibility of scale-up, should the production of S. uredinicola mycelium or metabolites as control agents be pursued. S. uredinicola 4149 was cultured in a New Brunswick Microferm fermenter, using 10 L of malt extract broth at 27°C, aerated at a rate of 4 L/minute, and stirred at 100 rpm. Periodic additions of sterile, distilled water were required to maintain the culture volume. The vessel was inoculated to give an initial culture density equal to that used in the growth curve shown in Fig. 17A. Twenty-mL samples were taken each day for 30 days, filtered for mycelial mass, and analyzed for maltol concentration and pH. The time course of maltol concentration had a maximum value of 33 µg/mL at 290 hours, and the medium pH trend closely resembled that of Fig. 17A. Scale-up therefore appeared to be feasible.

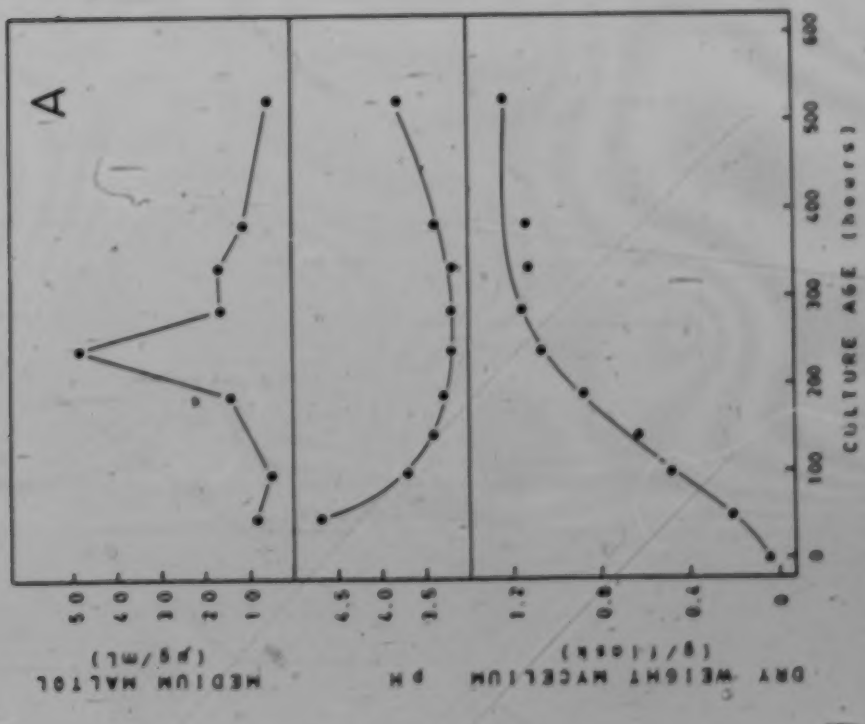
2) Examination of hyphal growth.

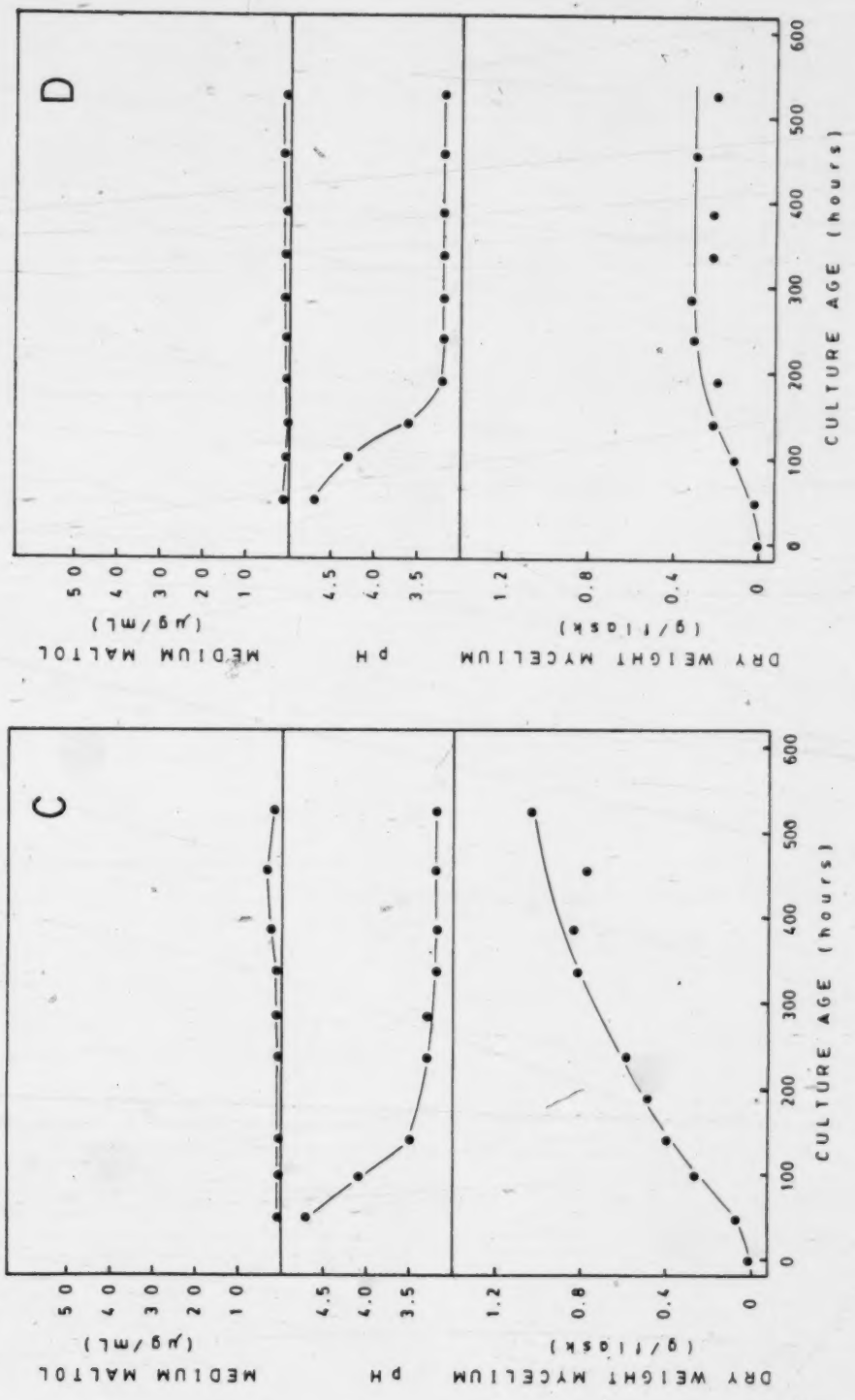
Growth rates of microbial cultures are usually interpreted as a multiple of the growth rate of a duplicating unit (culture growth rate = existing mass/ doubling time), that unit being the cell. The existence of a duplicating growth unit in cultures of filamentous fungi was first suggested by Plomley in 1959 (Trinci 1974); however, a number of irregularities arise when the growth of filamentous fungi is interpreted by this exponential model.

Figure 17. The effect of inoculum size on maltol production, pH and mycelial growth in S. uredinicola 4149 malt extract broth cultures.

Different volumes of a single inoculum preparation were used to produce 4 growth curves. The inoculum was a homogenate of 10 day old, malt extract broth culture (200 mL) as described in Materials and Methods. The inoculum contained 3.5 mg/mL dry weight of mycelium. The inoculum sizes for the data shown are: (A) 12 mL, (B) 8.0 mL, (C) 4.0 mL, and (D) 1.0 mL. The concentration of maltol contributed to the cultures from the inoculum was approximately 0.3 µg/mL of inoculum, and the amount present as a medium constituent was approximately 0.8 µg/mL.


B
MEDIUM MALTOL (μg/mL)
pH
DRY WEIGHT MYCELIUM (g/100ml)
CULTURE AGE (hours)


A
MEDIUM MALTOL (μg/mL)
pH
DRY WEIGHT MYCELIUM (g/100ml)
CULTURE AGE (hours)


C
D
DRY WEIGHT MYCELIUM (g/flask)
pH
MEDIUM MALTOL (μg/mL)
CULTURE AGE (hours)
0
100
200
300
400
500
600
0.4
0.8
1.2
3.5
4.0
4.5
10
20
30
40
50

It was therefore necessary to examine the growth of S. uredinicola at its most fundamental level of organization, the hypha, in order to gain insight into the factors contributing to the shape of its growth curve.

The late growth phase culture homogenate being used as inoculum was observed to be comprised largely of arthroconidia and short hyphal fragments resulting from disarticulation. A photomicrograph of a typical inoculum preparation is shown in Fig. 18. The rate of outgrowth from arthroconidia was examined first, assuming that the observations would approximate the initial growth of submerged cultures. Secondly, hyphal growth at colony margins was examined to insure that the basic ralationships observed for spore outgrowth were maintained in established mycelium.

Germination and outgrowth from spores was observed to be highly variable with respect to hyphal elongation rates, branching patterns, and septation. In general, germ tubes and branches attained a linear rate of extension very rapidly after their formation. The growth rates of the hyphae arising from a single spore, and their combined rate are shown in Fig. 19. Each branch appeares to extend at approximately the same rate. Fig. 20 shows photomicrographs of hyphal development from different spores.
Examination of hyphal growth at colony margins yielded data consistent with the outgrowth from spores: elongation rates

Figure 18. Photomicrograph of a typical S. uredinicola 4149 inoculum preparation.

An inoculum prepared by the standard procedure was photographed using phase-contrast microscopy. The preparation consisted of spores and hyphal fragments. Magnification is 330 x.

Figure 19. The extension of individual hyphae from a single spore, and their combined rates.

The outgrowth from a S. uredinicola spore was followed by taking time lapse photomicrographs at 1 hour intervals. The values plotted are total mycelial length (●), length of the main axis or germ tube (O), the first branch to form from the germ tube (■), and the second branch (▲). These three hyphae constituted the entire system, giving the total mycelial length.

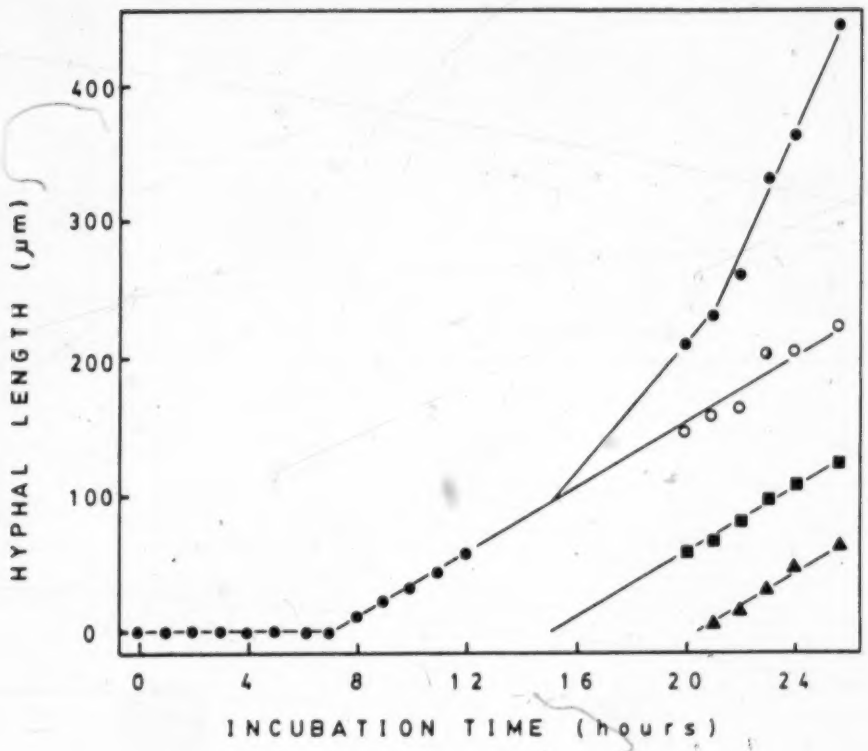

HYPHAL LENGTH (μm)
400
300
200
100
0
0
4
8
12
16
20
24
INCUBATION TIME (hours)

Figure 20. Photomicrograph showing the variability of outgrowth from individual spores of S. uredinicola 4149.

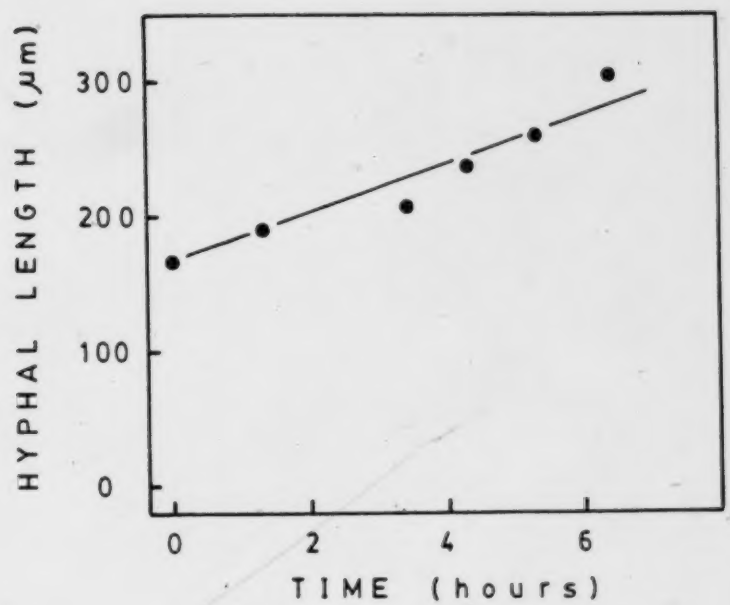
From the group of spores in the photomicrograph, it is apparent that outgrowth is highly variable with respect to the number of branches and distances between their origins on the main axes. Magnification is 330 x.

were linear at about 20 µm/hour, and a highly variable pattern of branching was recorded. Branch formation did not appear to be correlated with septation. Branches were initiated within the apical compartment, and at various locations between septa, although a tendancy towards initiation at the distal end of intercalary compartments, immediately behind the septum, was noted. The formation of more than one branch between regularly spaced septa was also observed. The distance between septa was highly conserved (mean ± SE = 27 ± 2 µm). In contrast to interseptal length, the mean distance between branches was greater and appeared to be more variable. One sample showed the mean ± SE of the distance between branches to be 41 ± 14 µm. Using the correlation coefficient ($r^2$), no correlation between intercalary compartment length and position relative to the hyphal tip was detected ($r^2$ = 0.006).

Fig. 21 shows the rate of extension of a typical hypha. In this experiment, no branches were initiated from the apical compartment (ahead of the first septum behind the tip). The apical compartment length varied between about 70 µm to 115 µm. In another series of observations a branch was initiated within the apical compartment of the main axis: initially the extension rates of the main axis and branch were slower than the final, linear rate that was ultimately attained by each tip. This was interpreted as an indication that a minimal hyphal length was required to

Figure 21. The extension rate of the main axis of an S. uredinicola hypha at a colony margin.

The rate of hyphal extension was determined from time lapse photomicrographs. The hypha examined was from a colony margin, and extended without branch initiation from the apical compartment during the period of observation.


HYPHAL LENGTH (μm)
300
200
100
0
0
2
4
6
TIME (hours)

support the maximal extension rate (the peripheral growth zone effect has been described by Fiddy and Trinci 1976B). Branch formation in older, septate regions of the main axis were also recorded. These data are represented graphically in Fig. 22. Fig. 23 shows photomicrographs of the hyphal system described in Fig. 22 at various time intervals: the branching system is rapidly obscured by mycelium from earlier branches and nearby hyphae. The same hyphal branching system has been redrawn schematically in Fig. 24 to illustrate the irregular nature of its development.

3) Culture growth rate in malt extract broth.

The early growth phase of S. uredinicola cultures were examined in order to study growth rates that were not greatly modified by nutrient depletion and metabolite accumulation in the medium. Dry weight determinations were made on cultures similar to those described in the experiment of Fig. 17A. The data used for kinetic analysis were collected at culture ages prior to any evidence of growth deceleration and before the change in medium pH exceded 1.0 units. The greatest mycelial yield in the data analyzed was about 1/3 of the maximum yield that the medium supported. Growth was thus assumed to procede at a rate virtually unaffected by environmental changes, and therefore was analagous to the mean, unrestricted growth of individual hyphae.

Figure 22. The extension rates of an S. uredinicola hyphal branching system at a colony margin.

The extension rates for a hypha and its branches at a colony margin were determined from time lapse photomicrographs. Plotted are the main axis (●) and the first branch, which arose from within the apical compartment (○). The second (△) and third (▽) branches to form along the main axis, both originating behind the first branch, are also plotted.

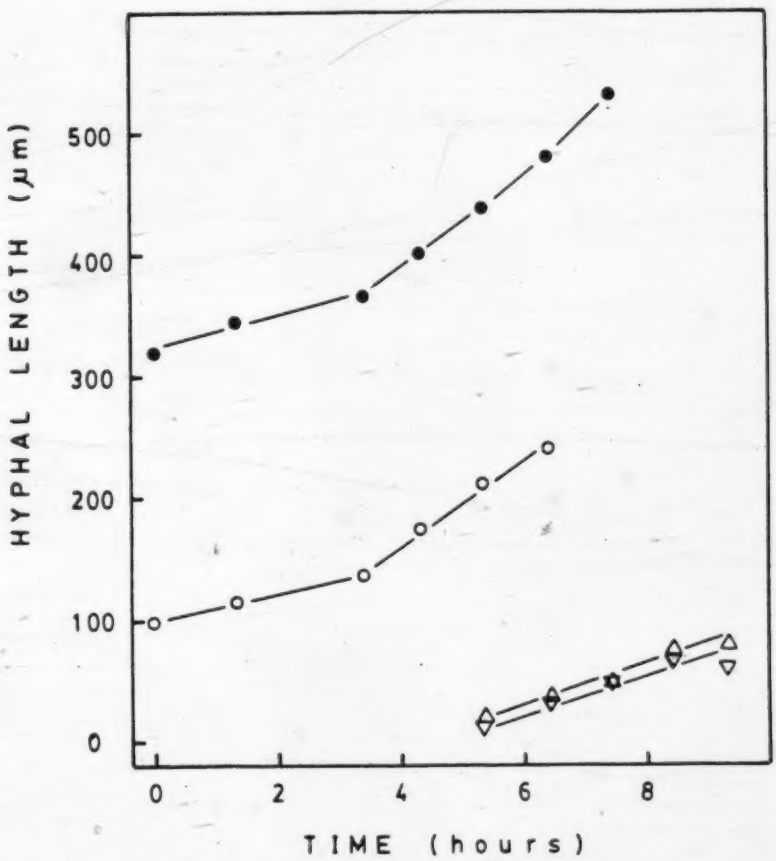
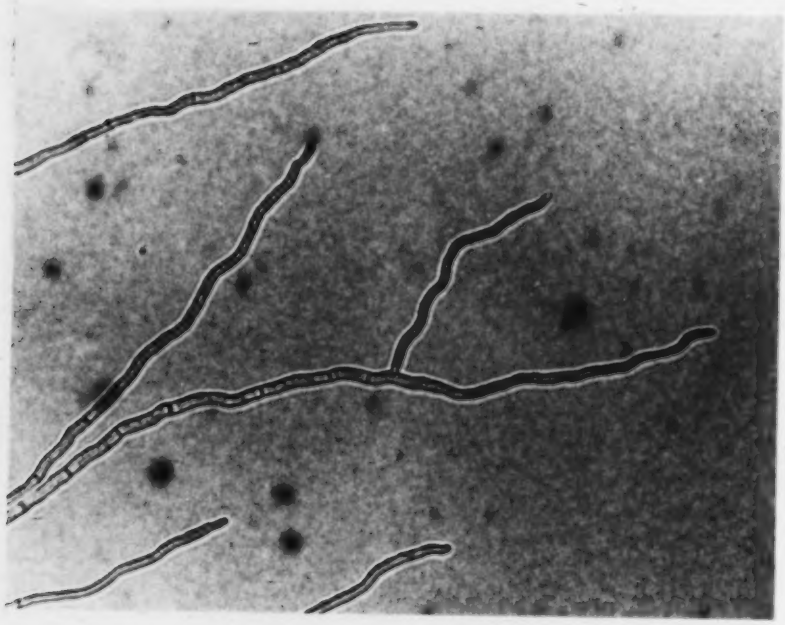

HYPHAL LENGTH (μm)
500
400
300
200
100
0
0
2
4
6
8
TIME (hours)

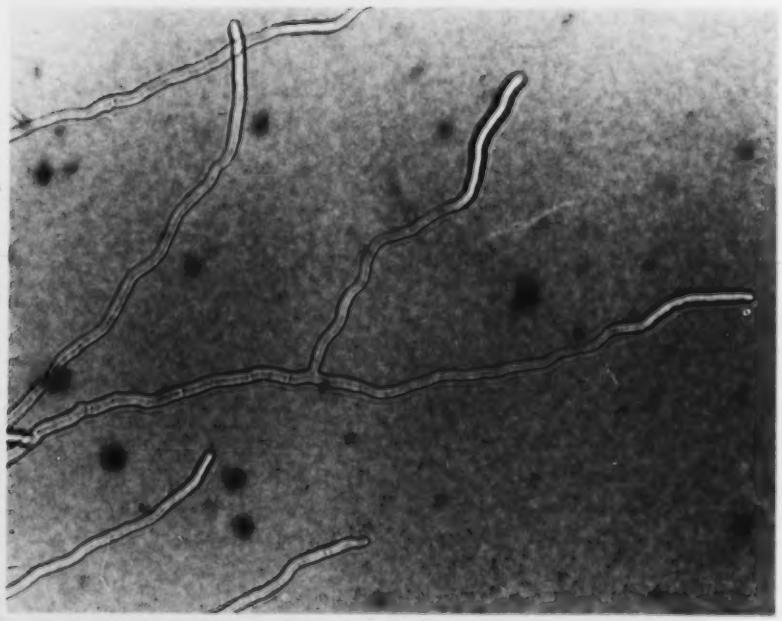
Figure 23. Photomicrographs of the hyphal branching system examined in figure 22.

The extension of a hypha and its branches at a colony margin were recorded by taking time lapse photomicrographs. The mycelium is shown at 0 hours (A), 5.3 hours (B), and 17.3 hours (C). Magnification is 330 x.

A



B

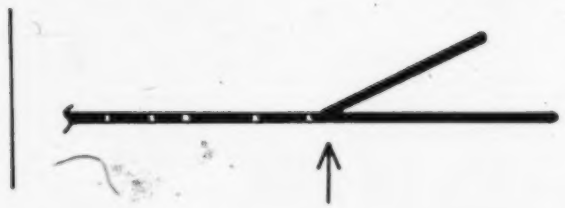


C

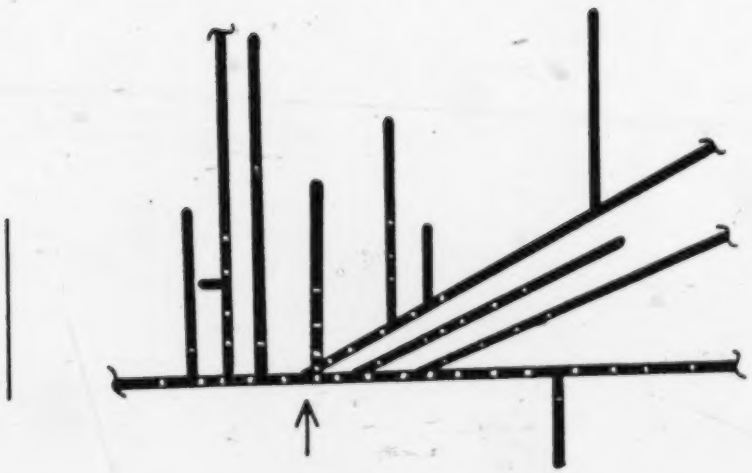
Figure 24. A schematic depiction of the hyphal branching system described in Figures 22 and 23.

The growth of a hypha and its branches at a colony margin were recorded by taking time lapse photomicrographs. The hyphal lengths are shown schematically to illustrate the irregular nature of the hyphal branching system. The diagrams for 0 hours (A) and 17.3 hours (B) incubation time are shown. The arrow locates the same position on each diagram. The bar represents 100μm. Septa are indicated by white spaces; however, some of the distal septa were not visible in the photomicrographs and therefore are not shown.

A



B



The data from this experiment are given in Fig. 25, plotted on linear scales to demonstrate that a regular, continuous function was observed. The variance between duplicate cultures was small, and was plotted separately for graphical clarity. Frequently the growth of filamentous fungi is interpreted as exponential, and doubling times are commonly stated. An alternate transformation, the cube root, is often used to linearize experimental data (Carroad and Wilke 1977).

In Fig. 26, three transformations of the growth curve data are given. The transformations are each based on one of the two general equations (equations 1 and 2) commonly used for fungal growth kinetics (Carroad and Wilke 1977).

$$dX/dt = \mu X \qquad (1)$$

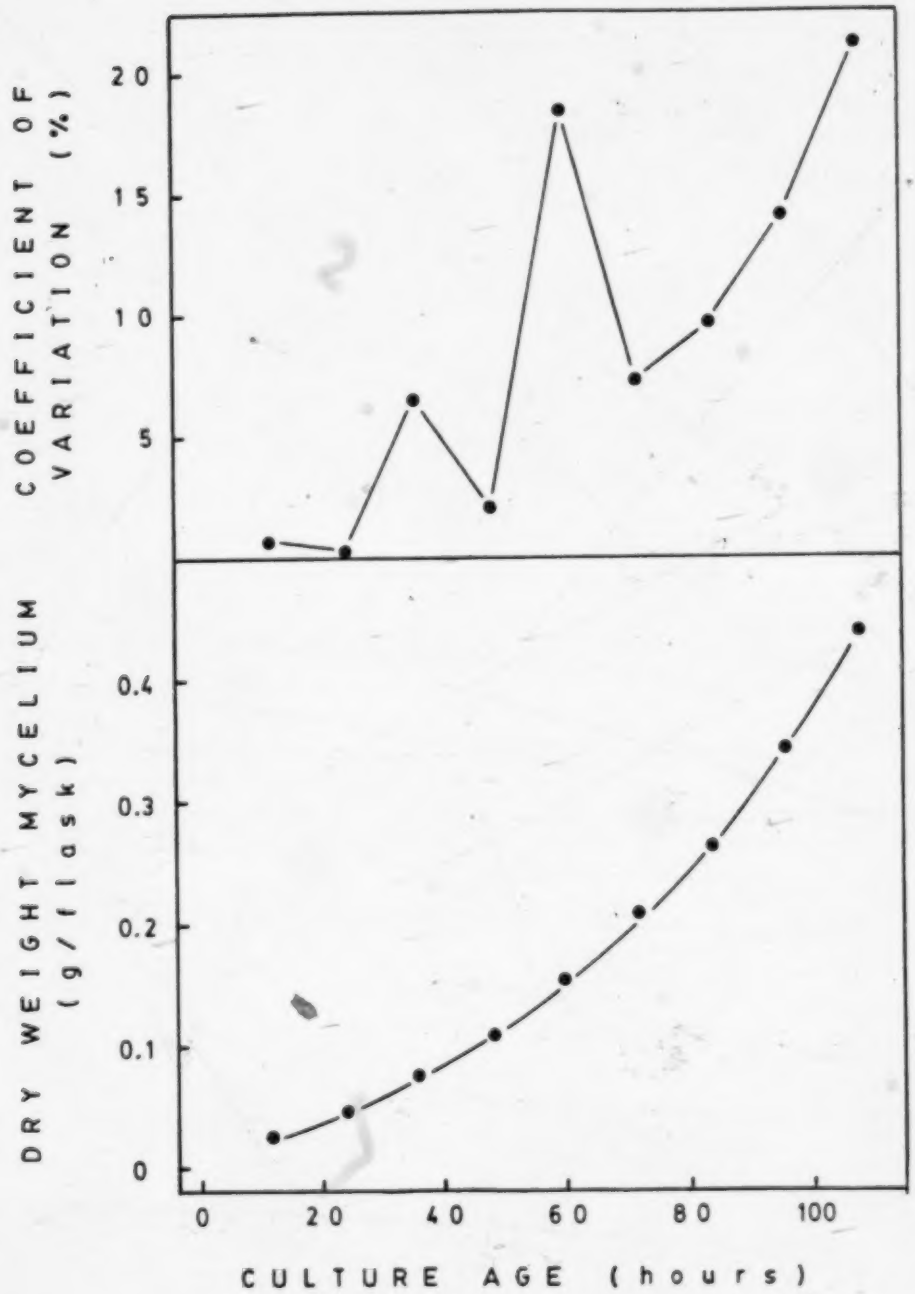
$$dX/dt = A X^{B} \qquad (2)$$

In equations 1 and 2, X represents mycelial mass, $\mu$ represents specific growth rate (i.e. doubling time$^{-1}$), t represents time, and A and B are constants. The transformations used for the three plots of Fig. 26 are logarithmic (assumed by equation 1), cube root (assumed by equation 2 with B = 2/3), and sixth root (assumed by equation 2 with B = 5/6).

The exponential and cube root relationships are

Figure 25. The early growth phase of S. uredinicola 4149 in malt extract broth.

S. uredinicola 4149 was grown in 500 mL Erlenmeyer flasks with 200 mL of malt extract broth. The cultures were incubated at 27°C with 200 rpm gyratory shaking. Dry weight was determined at approximately 12 hour intervals by harvesting 2 flasks. Variance between duplicates is given by the coefficient of variation, which is the standard deviation divided by the mean (expressed as percent).


COEFFICIENT OF VARIATION (%)
20
15
10
5
DRY WEIGHT MYCELIUM (g/flask)
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
CULTURE AGE (hours)

considered because they have been reported elsewhere (Emerson 1950), and the sixth root because it provided a reasonable transformation of the hypothetical model for hyphal growth considered in the Discussion. It is visibly evident from the plots that the cube root transformation provided the best linearization. This was proven by the coefficient of determination ($r^2$), which gives the proportion of the variance explained by the x,y relationship. The values of $r^2$ for each transformation of the data are given in table 2.

Table 2. Comparison of the models for growth of S. uredinicola in submerged cultures.

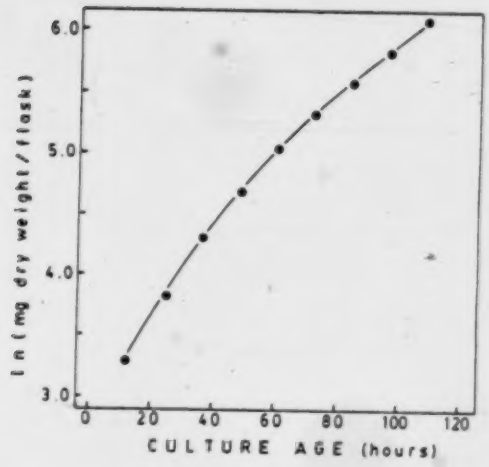
| Transformation | $r^2$ |
|---|---|
| logarithmic | 0.98 |
| cube root | 1.00 |
| sixth root | 0.99 |

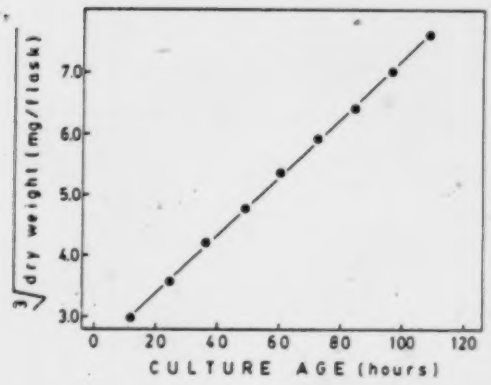
$r^2$ is the coefficient of determination

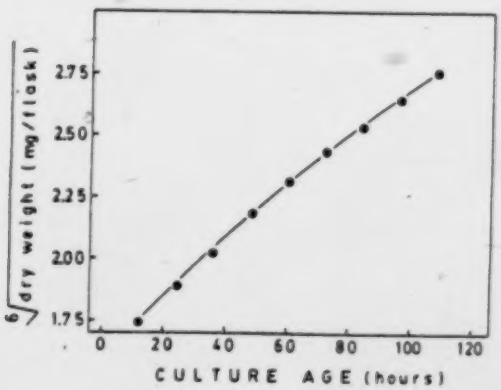
From the data presented here, it may be concluded that each transformation provides a close approximation of a complex function arising from the unique nature of hyphal growth. However, the possibility of a non-exponential

Figure 26. Logarithmic, cube root, and sixth root transformations of S. uredinicola 4149 growth in malt extract broth cultures.

S. uredinicola 4149 was grown in 500 mL Erlenmeyer flasks with 200 mL malt extract broth. Dry weight determinations were made at 12 hour intervals by harvesting 2 flasks. Growth was plotted as log, cube root, and sixth root of dry weight mycelium with time.


6.0
5.0
4.0
3.0
ln (mg dry weight/flask)
0
20
40
60
80
100
120
CULTURE AGE (hours)


7.0
6.0
5.0
4.0
3.0
$\sqrt[3]{\text{dry weight (mg/flask)}}$
0
20
40
60
80
100
120
CULTURE AGE (hours)


2.75
2.50
2.25
2.00
1.75
$\sqrt[6]{\text{dry weight (mg/flask)}}$
0
20
40
60
80
100
120
CULTURE AGE (hours)

growth curve may offer an explanation for the seemingly anomolous differences in nutritional requirements and growth rates which result from alteration of inoculum size.

4) Effects of inoculum size on synthetic medium cultures.

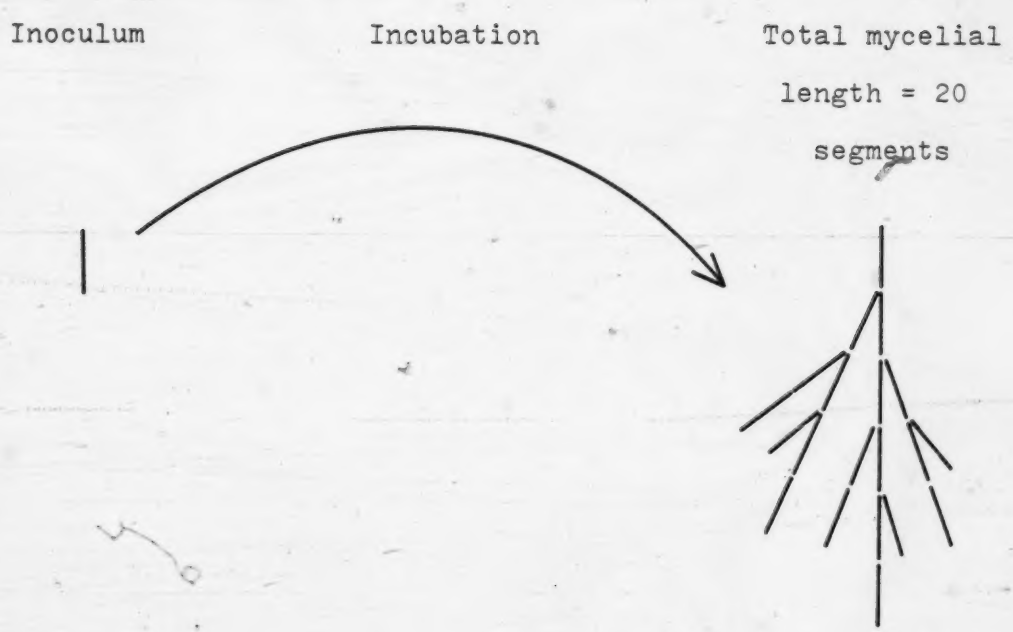
If inoculum-specific growth rates are in fact a manifestation of hyphal ontogeny, certain morphological differences are logically anticipated between cultures raised from small and large inocula. If growth at the level of individual hyphae is considered it would be expected that the mean hyphal length in either culture would be identical with respect to time, until other growth-affecting changes (i.e. oxygen transfer, nutrient depletion, and hyphal aggregation) became significant. Similarly, it may be expected that in order for a certain mass of mycelium to be produced, the individual hyphae from a small inoculum must show greater elongation of their main axis than those from a large inoculum. For clarity, this hypothesis is depicted schematically in Fig. 27.

Photomicrographs of S. uredinicola mycelium from large and small inoculum, synthetic medium cultures are shown in Fig 28. Mycelium at the same age from small (A) and large (B) inoculum cultures are juxtaposed; the large inoculum culture is approaching the end of the accelerating growth phase, whereas the small inoculum culture must undergo considerably more growth to reach that point. Mycelium from

Figure 27. Schematic depiction comparing equal total lengths of hyphae generated from different inoculum sizes.

In (A) the mycelium is generated from 1 growing apex; in (B) a five fold increase of inoculum is used. The hypothetical diagrams show growth occurring with each hyphal apex elongating at the same constant rate, and branches forming at a constant interval of hyphal length.

A:


Inoculum
Incubation
Total mycelial length = 20 segments

B:

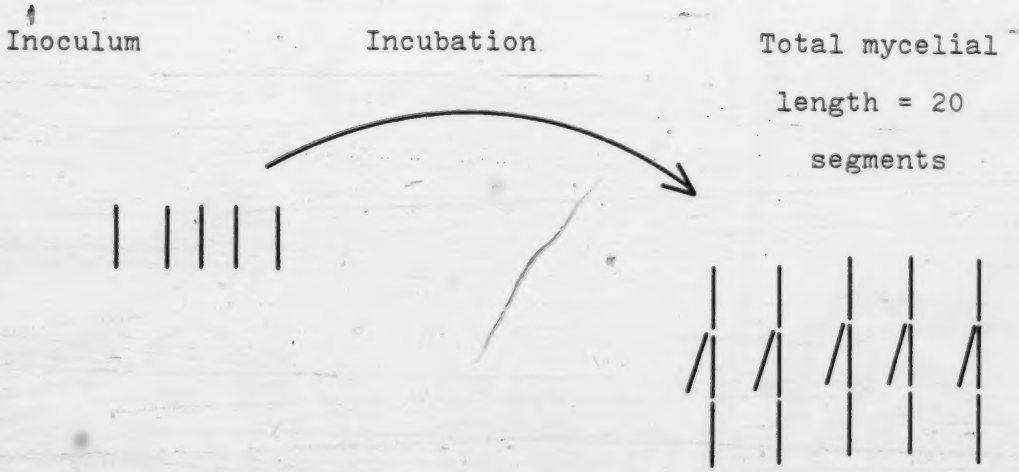
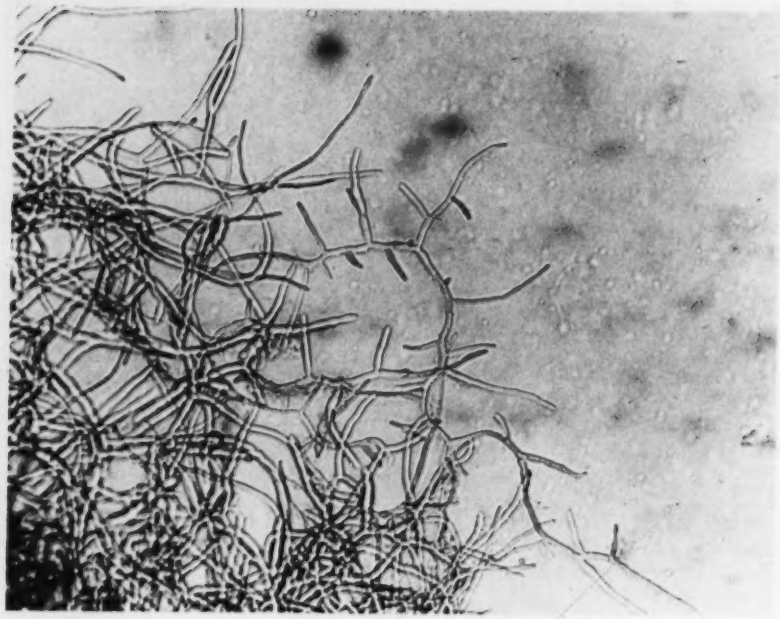

Inoculum
Incubation
Total mycelial length = 20 segments

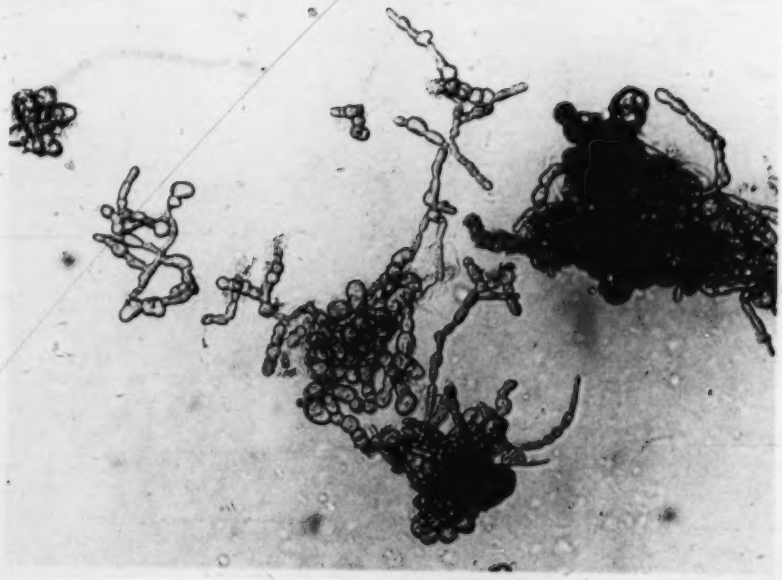
Figure 28. Photomicrographs of S. uredinicola mycelium from synthetic medium, submerged cultures.

Mycelium from small (A) and large (B) inoculum cultures is shown at the same culture age (287 hours). Also shown is mycelium at the same culture masses (approximately 0.2 grams dry weight per flask): the small inoculum culture (C) age is 359 hours, and the large inoculum culture (D) age is 145 hours. Magnification is 330 x.

A



B



C

D

the small inoculum culture is continuing to elongate without differentiation, but mycelium from the large inoculum has formed arthroconidia and begun to fragment. Also shown is mycelium from small (C) and large (D) inoculum cultures at a point on each growth curve where approximately the same mycelial mass has been achieved. Both are near the end of the accelerating growth phase. The small inoculum culture contains hyphae which are longer and have more extensive branching systems than the large inoculum culture. It is likely that these different morphologies partly account for the aggregation of hyphae and pellet formation which is promoted by decreased inoculum size (Metz et al. 1977).

In the above experiment, the small inoculum cultures were characterized by the formation of pellets, whereas dispersed mycelium occurred in the large inoculum cultures. The final appearance of each culture type is shown in Fig. 29. Pellet formation is known to introduce additional effects on the observed physiology of a culture, for example the consequences of limiting oxygen transfer (Ho et al. 1984), beyond the changes which might be expected to arise from hyphal differentiation. In order to design a medium in which maltol production may be studied, the degree to which nutrition is affected by inoculum size must be explored.

Since the mathematical functions used to describe the growth of fungi are empirical, a systematic method of dividing the curve into phases is needed. The graph shown

Figure 29. The final appearance of mycelium from large and small-inoculum cultures.

Mycelium from a large -inoculum culture (A) and a small-inoculum culture (B) are shown at 2.1 x magnification. Samples of each culture in 9 cm petri dishes are also shown (C); the top sample is from a large-inoculum culture, and the bottom is from a small inoculum culture (magnification is 0.83 x).

in Fig. 30 was used to identify the point at which the accelerating growth phase ends. The mass of new mycelium formed increased with each time interval until the end of the accelerating growth phase was reached. Evidence that this point roughly coincides with the end of unrestricted growth can be obtained by plotting the mass of mycelium formed against inoculum size. Figs. 31A and 31B show this analysis at culture ages before and after changes in the medium begin to affect growth. During unrestricted growth, the fungal mass produced was in direct proportion to the inoculum size such that the line extrapolated to 0,0 (Fig. 31A). As the faster growing, large inoculum cultures began to deplete the medium and growth rates declined, the relationship began to decay as shown in Fig. 31B.
It is necessary to distinguish between the period of unrestricted growth and decelerating growth, so that physiological effects arising from changes in the medium may be distinguished from inherent qualities of the mycelium. The ratio of carbohydrate to nitrogen utilization is used to detect trends in substrate requirement which may affect growth and secondary metabolite production.

In an experiment where the removal of total carbohydrate and nitrogen sources was measured in growth curves generated by eight different inoculum sizes, it was found that the ratio of carbohydrate to nitrogen removed from the medium increased with both culture age and inoculum

Figure 30. Graph showing new mycelium formed within time intervals, for large and small-inoculum cultures.

S. uredinicola 4149 was cultured in 200 mL of synthetic medium in 500 mL Erlenmeyer flasks (incubated at 27°C with 200 rpm gyratory shaking). Each point indicates the end of an approximately 72 hour time interval; the mass of mycelium generated within that time interval ($t_2 - t_1$) is shown for small-inoculum cultures (relative size = 1) and large inoculum cultures (relative size = 10).

The large-inoculum cultures are represented by (●), and the small-inoculum cultures by (▲).

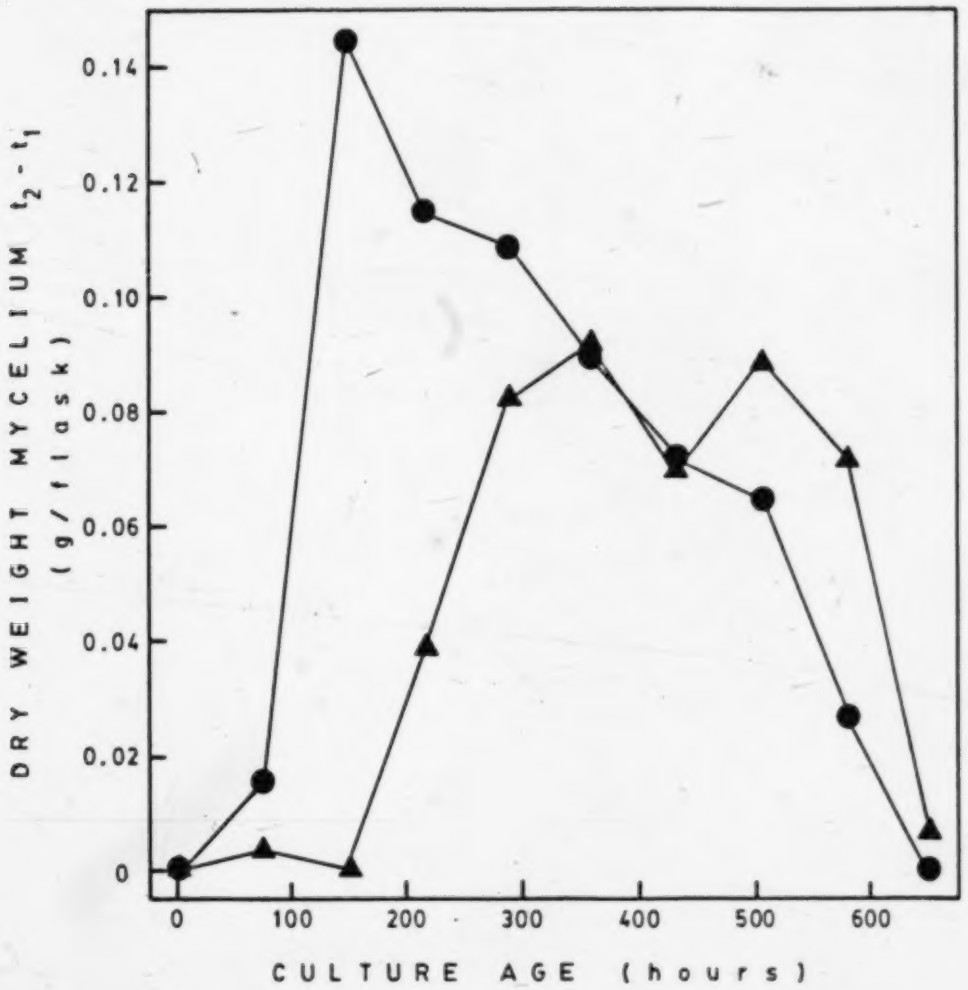
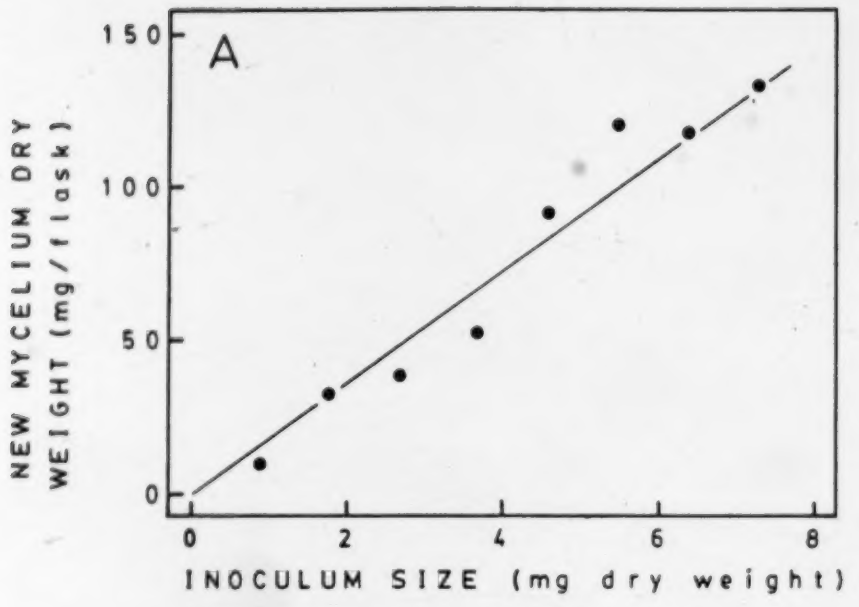
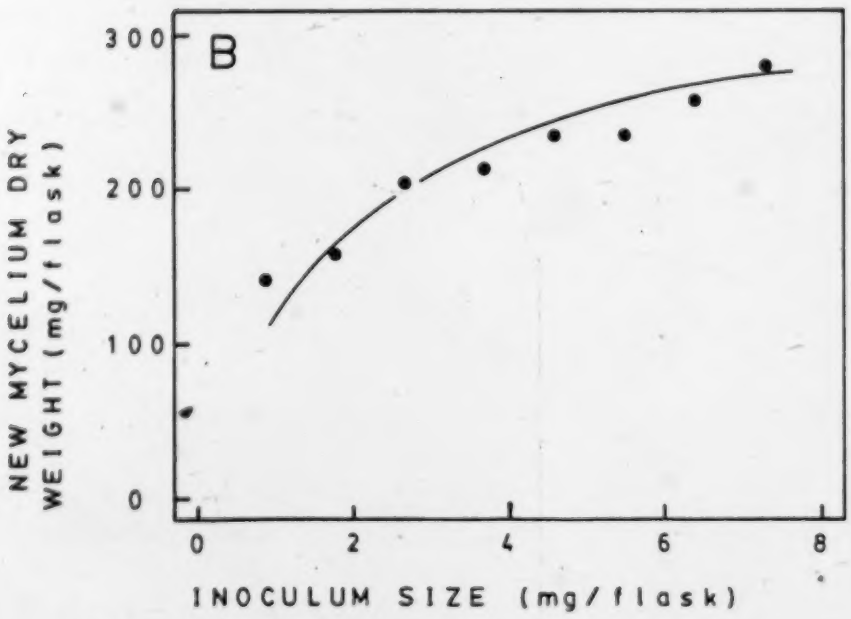

DRY WEIGHT MYCELIUM $t_2 - t_1$ (g/flask)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
100
200
300
400
500
600
CULTURE AGE (hours)

Figure 31. The relationship between new mycelial mass and inoculum size.

S. uredinicola was cultured in 500 mL Erlenmeyer flasks with 200 mL of synthetic medium. The new mycelium formed (total mycelial mass of the culture less the inoculum mass) is shown at 213 hours (A) and at 360 hours (B), for each of 8 inoculum sizes.

In (A) the mycelial yield is in direct proportion to inoculum size. In (B) the large inoculum cultures are approaching maximum mycelial yield.


A
150
100
50
0
0
2
4
6
8
NEW MYCELIUM DRY WEIGHT (mg/flask)
INOCULUM SIZE (mg dry weight)


B
300
200
100
0
0
2
4
6
8
NEW MYCELIUM DRY WEIGHT (mg/flask)
INOCULUM SIZE (mg/flask)

size. The results of this experiment are shown in Fig. 32. In this experiment, the end of the unrestricted growth phase in the fastest growing culture was approximately 213 hours. At that time the range in the carbohydrate to nitrogen ratio was from 0.17 to 3.18, showing a positive correlation with inoculum size. At 360 hours, the ratio ranged from 5.9 to 12.5, also showing a significant positive correlation. Because the effect is observed before and after the end of the accelerating growth phase, it is interpreted as a quality of the mycelium rather than a consequence of changing medium conditions.

In the above experiment, the medium contained 10 g/L glucose and 0.07 g/L $NH_4NO_3$. After maximum mycelial mass was reached, all cultures contained residual carbohydrate (from 6.5 mM to 22 mM glucose) and residual ammonium (from 5.41 mM to 5.92 mM). In both cases the high values occurred in small inoculum cultures. No utilization of nitrate occurred which indicated that ammonium was not limiting. The final pH of the medium was 3.0 to 3.1 in both cases.

Growth curves from small (1 x) and large (10 x) inocula showing the glucose and ammonium content of the medium, pH and mycelial dry weight with time are presented in Fig. 33. In this experiment, the defined medium was modified by replacing $NH_4NO_3$ with an equimolar amount of $NH_4Cl$. The final pH observed in both cultures was 2.6 which may have had a significant effect on late growth rates. The rate of

Figure 32. The ratio of carbohydrate to nitrogen removed from synthetic medium by S. uredinicola 4149 as a function of inoculum size.

S. uredinicola 4149 was cultured in 500 mL Erlenmeyer flasks with 200 mL of synthetic medium. The flasks were incubated at 27°C with 200 rpm gyratory shaking. The amount of glucose and nitrogen used by the fungus was determined from the concentrations remaining in the medium. The ratio of glucose to nitrogen used is plotted as a function of inoculum size for values determined at 213 hours (A) and 360 hours (B) culture age. The accelerating growth phase of the fastest growing cultures (largest inoculum) ended at about 213 hours.

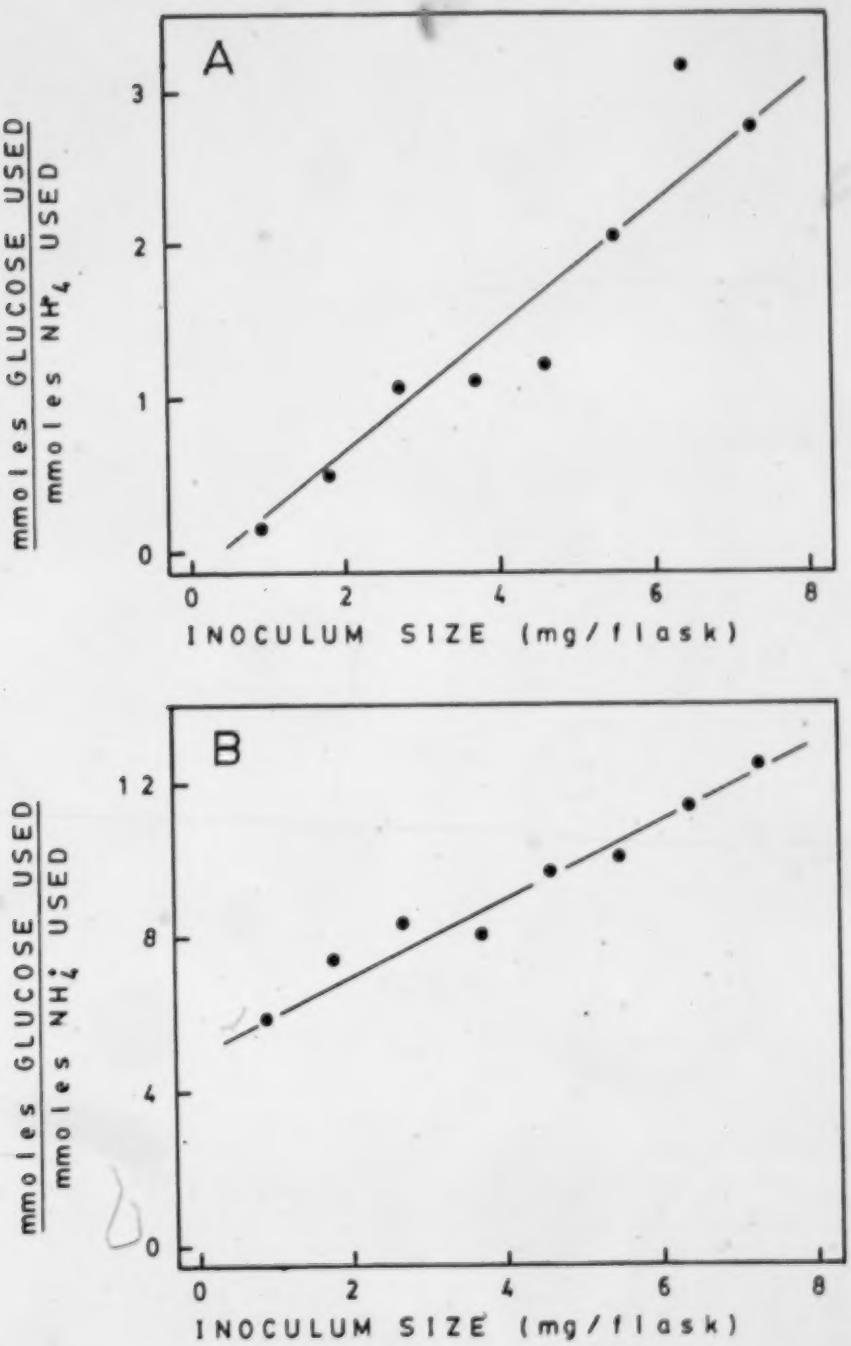
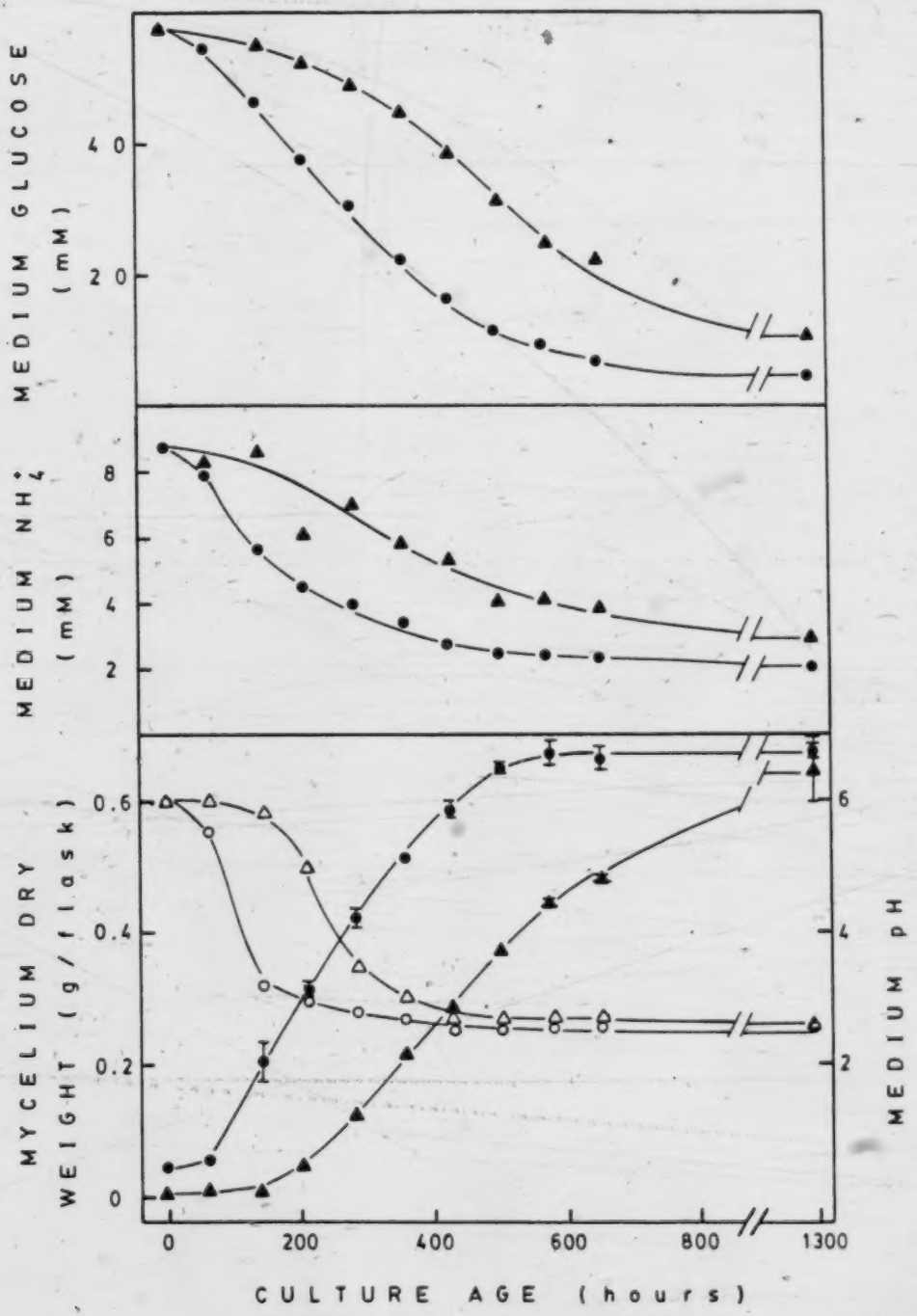

A
mmoles GLUCOSE USED / mmoles NH4 USED
0
1
2
3
0
2
4
6
8
INOCULUM SIZE (mg/flask)
B
mmoles GLUCOSE USED / mmoles NH4 USED
0
4
8
12
0
2
4
6
8
INOCULUM SIZE (mg/flask)

Figure 33. Growth curves of small and large-inoculum cultures showing pH, medium glucose, medium ammonium, and dry weight.

S. uredinicola 4149 was cultured in 500 mL Erlenmeyer flasks with 200 mL of synthetic medium. The flasks were incubated at 27°C with 200 rpm gyratory shaking. The cultures were inoculated with large (10 x) or small (1 x) inocula. Duplicate flasks were harvested at 2 day intervals and assayed for dry weight, glucose, and ammonium (large inoculum ●, small inoculum ▲); open symbols are used for pH. Error bars represent standard error of the mean.


MEDIUM GLUCOSE (mM)
40
20
MEDIUM NH4+ (mM)
8
6
4
2
MYCELIUM DRY WEIGHT (g/flask)
0.6
0.4
0.2
0
MEDIUM pH
6
4
2
0
200
400
600
800
1300
CULTURE AGE (hours)

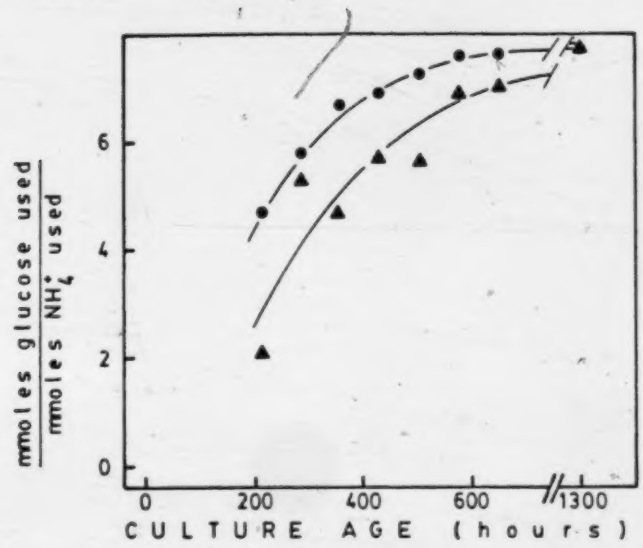
culture volume loss due to evaporation was 0.42 mL per day and was accounted for in the calculations. The replots in Fig. 34 show the glucose/ammonium utilization ratio for the two inoculum size cultures as a function of culture age, and as a function of total new mycelium. The glucose/ammonium utilization ratio increases with both mass and age of the mycelium. The ratio tends towards a maximum with respect to age and shows a difference between inoculum sizes; there is no apparent difference in glucose/ammonium utilization between the two cultures with respect to mass.

The difference between the glucose/ammonium utilization rates with respect to culture age for the large and small inocula may be explained by the relationship between the two growth curves: the large inoculum cultures produce a curve which is on a compressed time scale compared to the small inoculum cultures. This effect is revealed by plotting the time interval between the two cultures at equal values of mycelial mass, as a function of the age of the large inoculum culture. This data is shown in Fig. 35. From the linear, positive correlation, it may be calculated that a 49% reduction of the small inoculum culture age scale will superimpose that curve on the large inoculum curve. In contrast, exponential curves generated from different inoculum sizes are separated by a constant time interval determined by inoculum size and specific growth rate. That is, a plot such as Fig. 35 would have zero slope for

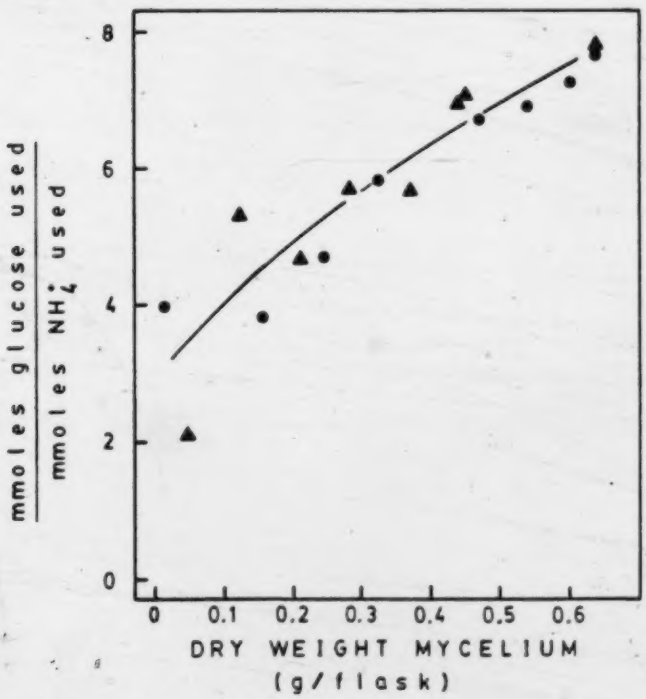
Figure 34. The ratio of glucose to ammonium used by small and large-inoculum cultures as a function of culture age, and total new mycelium.

S. uredinicola 4149 was cultured in 500 mL Erlenmeyer flasks with 200 mL of synthetic medium. The flasks were incubated at 27°C with 200 rpm gyratory shaking. Flasks of large inoculum (10 x, ●) and small inoculum (1 x, ▲) cultures were harvested at 2 day intervals. Glucose and ammonium used was determined from the concentrations remaining in the medium, and the ratio of glucose to ammonium used was plotted as a function of culture age (A) and of mycelial mass (B). Each point is the mean of 2 flasks.

A


mmoles glucose used / mmoles NH4+ used
0
2
4
6
0
200
400
600
1300
CULTURE AGE (hours)

B


mmoles glucose used / mmoles NH4+ used
0
2
4
6
8
0
0.1
0.2
0.3
0.4
0.5
0.6
DRY WEIGHT MYCELIUM
(g/flask)

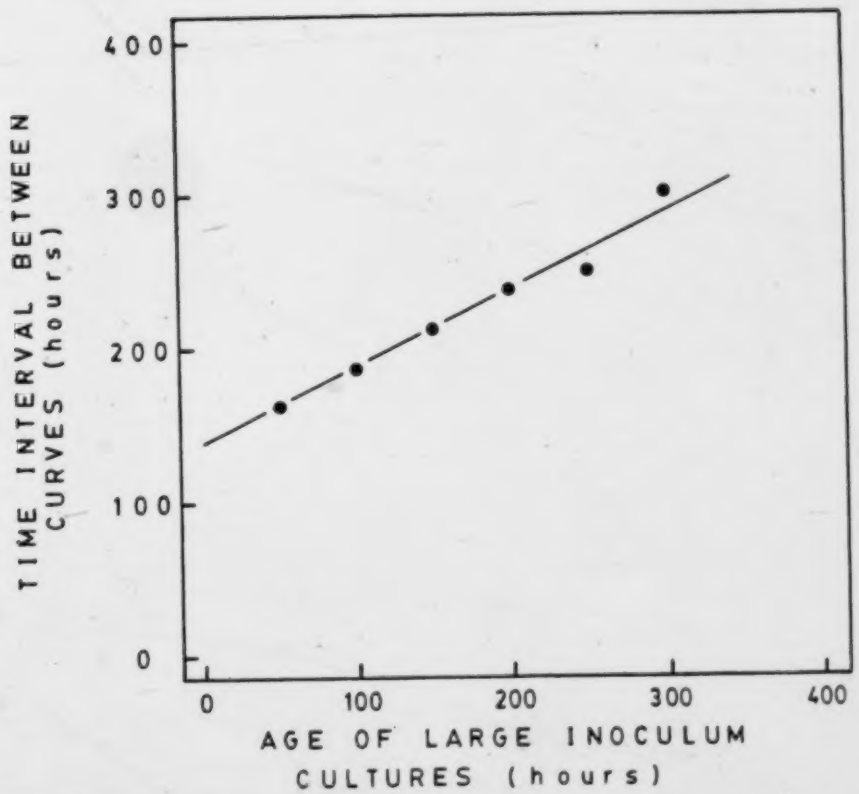
exponential growth curves. The amount of both glucose and ammonium removed from the medium by small inoculum (1 x) cultures in achieving a mycelial mass equal to the large inoculum (10 x) is approximately 4% of the total available. Effects on growth rate arising from this source are presumed to be negligible.

The relationship shown in Fig. 35 may therefore be interpreted as being a consequence of a non-exponential growth function, and it serves to illustrate the dependance of culture growth rate on inoculum size. These kinetics are consistent with the growth rates which occur when growth is interpreted by the general equation (2) in the previous section. By comparison, exponential growth rate is mathematically independant of inoculum size.

A consideration arising from the altered time-scale effect is the prolonged exposure of mycelium from a small inoculum culture to certain medium conditions. For example, in Fig. 33 the large inoculum culture reaches maximum mycelial yield 480 hours after the pH falls below 4.0, whereas the small inoculum culture requires greater than 600 hours. The composition of the medium changes continuously with growth, and the effect of prolonged exposure on secondary metabolite production is not known. It was observed that low levels (less than 1 µg/mL) of maltol first appeared in these defined-medium cultures at the 579 hour time point.

Figure 35. The time interval separating the growth curves of small and large-inoculum cultures.

S. uredinicola 4149 was grown in 500 mL Erlenmeyer flasks with 200 mL of synthetic medium. Cultures were inoculated with large (10 x) or small (1 x) inoculum size. The culture age difference between equal mycelial masses on the two growth curves (time interval between curves) is plotted as a function of the large inoculum culture age.

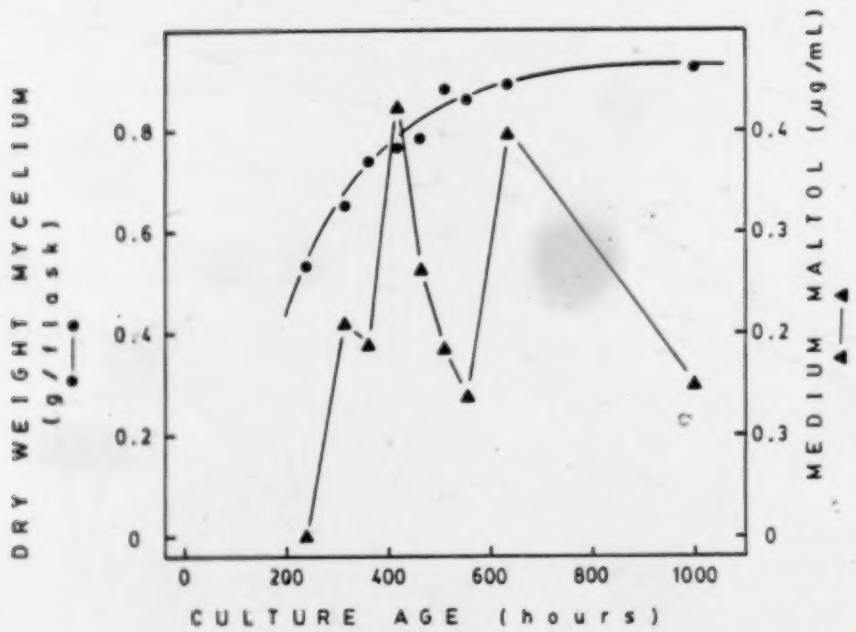

TIME INTERVAL BETWEEN CURVES (hours)
400
300
200
100
0
0
100
200
300
400
AGE OF LARGE INOCULUM CULTURES (hours)

Specific ammonium utilization tended to decrease with age and mycelial mass while specific glucose utilization remained relatively constant. Much larger changes were observed in the early growth phase than in the middle and late growth phase; however, insufficient data was collected for any definite conclusions to be drawn. The carbon/nitrogen utilization ratio was used here principally to make qualitative distinctions between cultures. The data accumulated provided a means for selecting fungal morphology and time course effects on medium composition.

The final experiment of this series was designed to follow the time course of maltol production in synthetic medium. The basal synthetic medium was modified by doubling the glucose concentration to 20 g/L to ensure that glucose was not limiting. Also, the phosphate concentration was doubled to 19.34 mM to increase the buffering capacity of the medium. The same concentration of $NH_4Cl$ was used as in the preceding experiment. The data presented in Fig. 36 shows that only very low levels of maltol were produced, and approximately the same time course of production as seen in malt extract broth cultures was observed. The medium pH dropped to 2.7 and is likely a consequence of supplying ammonium as the sole nitrogen source.

Figure 36. Maltol production by S. uredinicola in synthetic medium.

S. uredinicola 4149 was cultured in 500 mL flasks containing 200 mL medium, incubated at 27°C with 200 rpm gyratory shaking. The medium contained 20 g/L glucose, 1.14 g/L $NH_4Cl$, and 19.34 mM phosphate.


DRY WEIGHT MYCELIUM (g/flask)
MEDIUM MALTOL (µg/mL)
0
0.2
0.4
0.6
0.8
0.3
0.4
0
200
400
600
800
1000
CULTURE AGE (hours)

Biological Effects of Maltol

1) Conditions for E. harknessii spore germination.

The spore germination assay used to detect inhibitory activity in the purification procedures was conducted under the published conditions for optimal germination (Fairbairn et al. 1983, Powell and Morf 1966). In this section a quantitative assay of greater precision was required; therefore, the slide method was used, and the optimal conditions for germination were established.

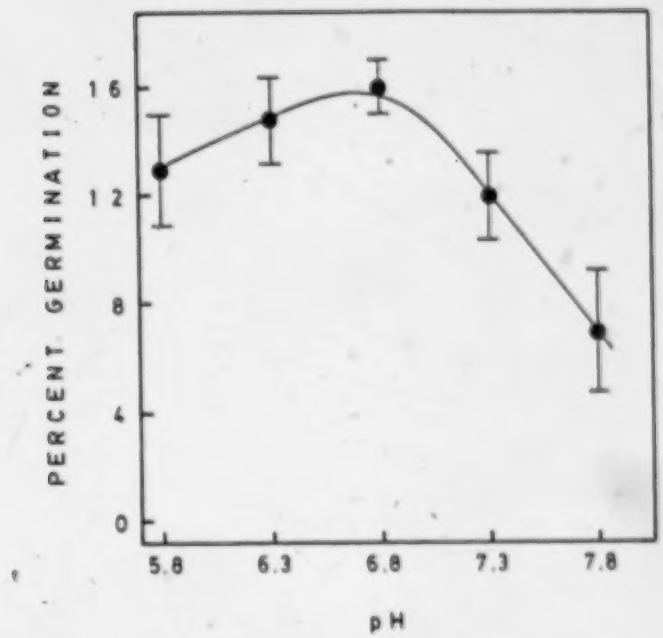
The optimal pH was determined using 20 mM potassium phosphate at pH 5.8 to 7.8 in intervals of 0.5 pH units. It was observed that maximum germination occured at pH 6.8, and greater inhibition of germination was caused by higher values than lower values. The response curve is shown in Fig. 37. The concentration of phosphate is known to affect spore germination in other systems (Bell and Dally 1962); therefore, germination was assayed using phosphate concentrations in 20 mM intervals at pH 6.8. The dose-response to phosphate, shown in Fig. 38, indicates that concentrations above approximately 40 mM began to suppress germination. In some instances the compounds tested as germination inhibitors were sufficiently acidic or basic to exceed the buffering capacity of the germination medium, and neutralization with NaOH or HCl was required. The effect of the counterions was investigated by conducting spore germination assays in the presence of NaCl, with 10 mM

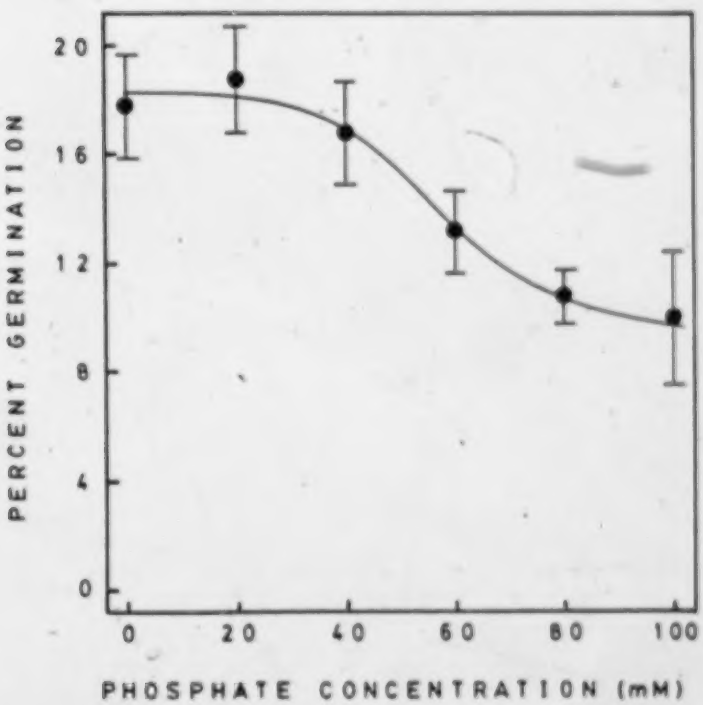
Figure 37. The response of E. harknessii germination to pH.

Germination was assayed by the slide method using 2% water agar with 20 mM potassium phosphate. Bars represent standard error of the mean.

Figure 38. The dose-response of E. harknessii spore germination to phosphate concentration.

Germination was determined by the slide mithod using 2% water agar at pH 6.8. Bars represent standard error of the mean.


PERCENT GERMINATION
0
4
8
12
16
5.8
6.3
6.8
7.3
7.8
pH


PERCENT GERMINATION
0
4
8
12
16
20
0
20
40
60
80
100
PHOSPHATE CONCENTRATION (mM)

phosphate at pH 6.8. No effect was noted, using concentrations up to 40 mM.

2) Effect of maltol and other compounds on E. harknessii germination.

A dose-response curve of E. harknessii spore germination in the presence of maltol revealed that 0.1 mg/mL maltol caused approximately a 76% decrease in germination. The response curve is shown in Fig. 39. Glutamic acid has been reported to reverse the antibacterial effect of maltol (Banarjee et al. 1980); therefore, the effect of glutamic acid on the inhibition of E. harknessii spores by 0.3 mg/mL maltol was examined. In contrast to the published effect, it was found that the addition of 0.2 mg/mL glutamate resulted in a statistically significant reduction of germination in the presence of maltol. In the absence of maltol, up to 1.2 mg/mL glutamic acid had no significant effect on germination. The results of this experiment are shown in Fig. 40.

Maltol has also been found to act as an iron transport agent for Salmonella typhimurium (Akers et al. 1980). Its chelating ability suggested a relationship with the $Ca^{++}$ and phosphate controlled protease activity required for spore germination in Microsporium (Page and Stock 1971). Therefore, the effect of 0.3 mg/mL maltol in the presence of equimolar $CaCl_2$ was tested on germination using water agar

Figure 39. The dose-response of E. harknessii spore germination to maltol.

Germination was determined by the slide method using 10 mM potassium phosphate at pH 6.8, and graded concentrations of maltol. Bars represent standard error of the mean.

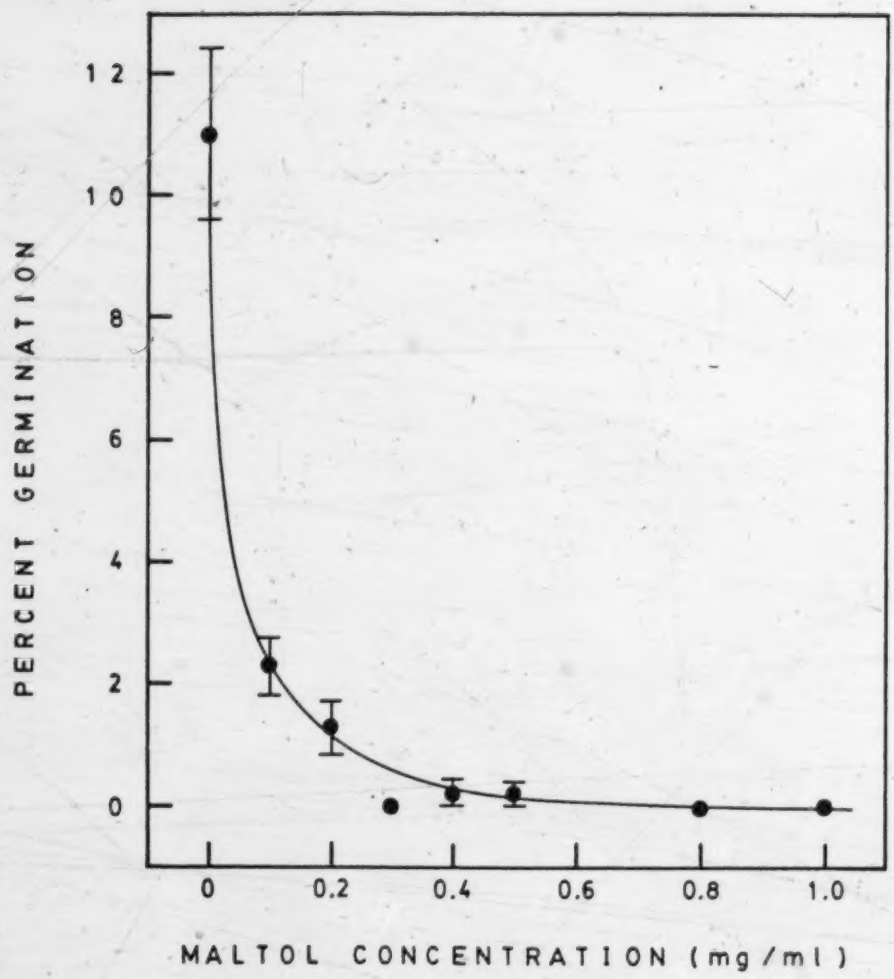
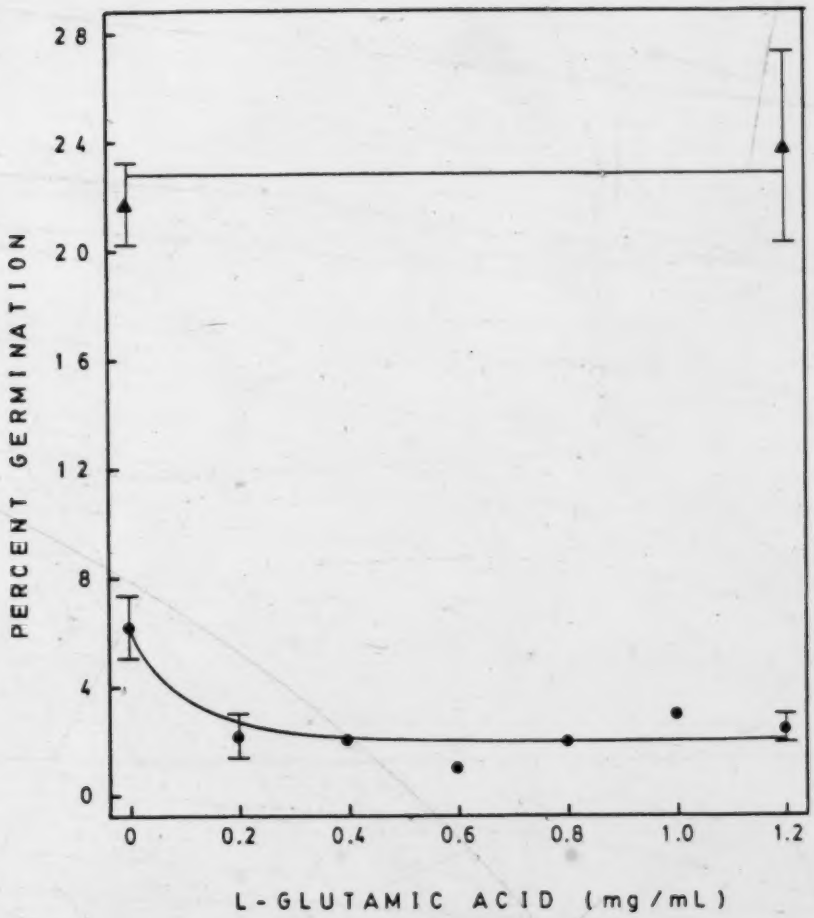

PERCENT GERMINATION
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1.0
MALTOL CONCENTRATION (mg/ml)

Figure 40. The effect of glutamic acid on E. harknessii spore germination in the presence of maltol

Germination in the presence (●) and absence (▲) of 0.30 mg/mL maltol at graded concentrations of L-glutamic acid (neutralized with NaOH) was determined by the slide method. Bars represent standard error of the mean.


PERCENT GERMINATION
28
24
20
16
12
8
4
0
0
0.2
0.4
0.6
0.8
1.0
1.2
L-GLUTAMIC ACID (mg/mL)

without phosphate buffer; no reversal of the inhibition due to maltol was observed. An increase in germination was observed in the presence of $CaCl_2$ alone, but due to large variance between replicates this observation was not statistically significant.

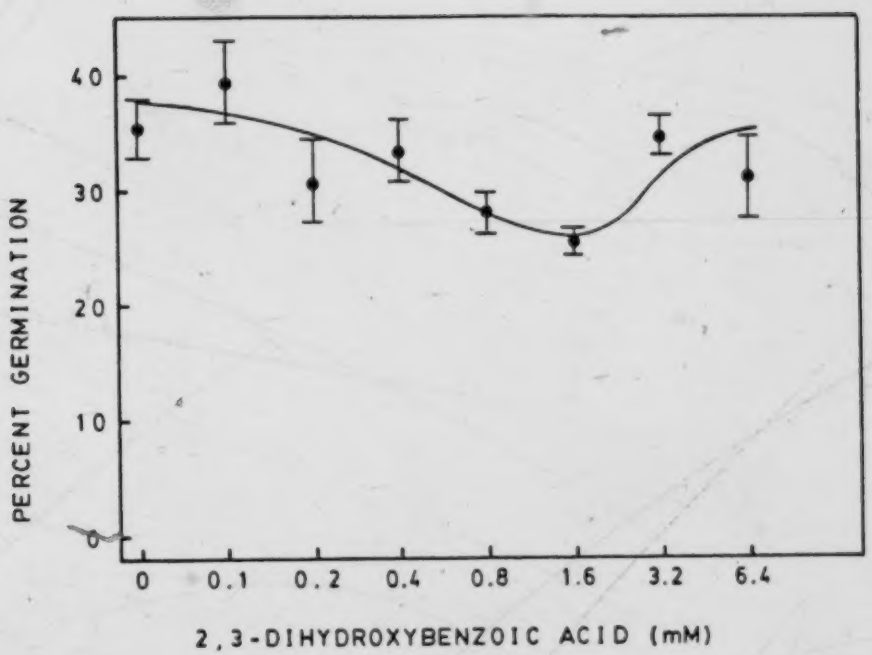
Maltol has been described as a non-competitive inhibitor of glyoxalase I activity ($K_i = 4 \times 10^{-4}$ M; Douglass and Nadvi 1979); at this concentration maltol also caused a significant reduction of E. harknessii spore germination. Other inhibitors of glyoxalase I reported in the same study were 2,3-dihydroxybenzoic acid, rhodizonic acid, and squaric acid. These compounds were therefore tested for their ability ot inhibit E. harknessii spore germination. An unusual effect was observed with rhodizonic acid: at all concentrations shown in Fig. 42, complete inhibition of germination occurred under the center of the coverslips, but spores near the edges or not covered reached control level germination. Germination was measured by counting along a straight line towards the center of the coverslips, starting from the edge. In this way the anomolous effect between and within each quadruplicate set of slides was randomized. The percent germination reported does not provide an accurate measure of the effect of rhodizonic acid, but nonetheless confirms a dose-response relationship. The coverslip effect did not occur in the controls or with other compounds. Possibly exposure to air

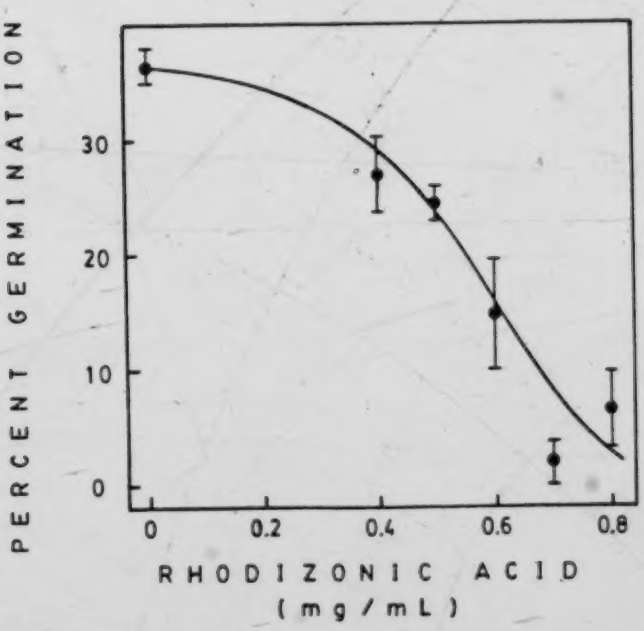
Figure 41. The dose-response of E. harknessii spore germination to 2,3-dihydroxybenzoic acid.

Germination was determined by the slide method using graded concentrations of 2,3-dihydroxybenzoic acid (neutralized with NaOH). Bars represent standard error of the mean.

Figure 42. The dose-response of E. harknessii spore germination to rhodizonic acid.

Germination was determined by the slide method using graded concentrations of rhodizonic acid (neutralized with NaOH). Bars represent standard error of the mean.


PERCENT GERMINATION
40
30
20
10
0
0
0.1
0.2
0.4
0.8
1.6
3.2
6.4
2,3-DIHYDROXYBENZOIC ACID (mM)


PERCENT GERMINATION
30
20
10
0
0
0.2
0.4
0.6
0.8
RHODIZONIC ACID
(mg/mL)

affects either the chemistry of the compound or its biological activity, but this was not investigated further. 2,3-Dihydroxybenzoic acid caused a slight reduction in germination at 1.6 mg/mL; however, its effect did not increase with higher concentrations. The response curve is shown in Fig. 41. No inhibition of germination was observed with squaric acid at concentrations up to 0.5 mg/mL.

3) Maltol as an inhibitor of mycelial growth. -

To assess the effect of maltol on mycelial growth, a number of wood decay organisms were tested. The concentration to be used was selected by determining the dose-response relationship against Ceratocystis ulmi. In Fig. 43 it can be seen that approximately 50% decrease in mycelial yield of C. ulmi is caused by 0.55 mg/mL maltol. At that maltol concentration other wood decay fungi showed variable responses; these data are given in Table 3. Also included in this experiment was S. uredinicola 4149, the strain from which maltol was isolated. The sensitivity of this strain to maltol is typical of the organisms tested, and therefore a limit to its ability to produce maltol in antifungal concentration exists.

4) Survey of yeast sensitivities to maltol.

A variety of yeasts were screened for maltol sensitivity with the aim of obtaining convenient, sensitive

Figure 43. The dose response of Ceratocystis ulmi to maltol.

C. ulmi was grown in 50 mL of malt extract broth in 125 mL Erlenmeyer flasks at 27°C with 200 rpm gyratory shaking. Graded amounts of maltol were included in the medium, and mycelial dry weight was determined after 29 hours incubation. Bars represent standard error of the mean.

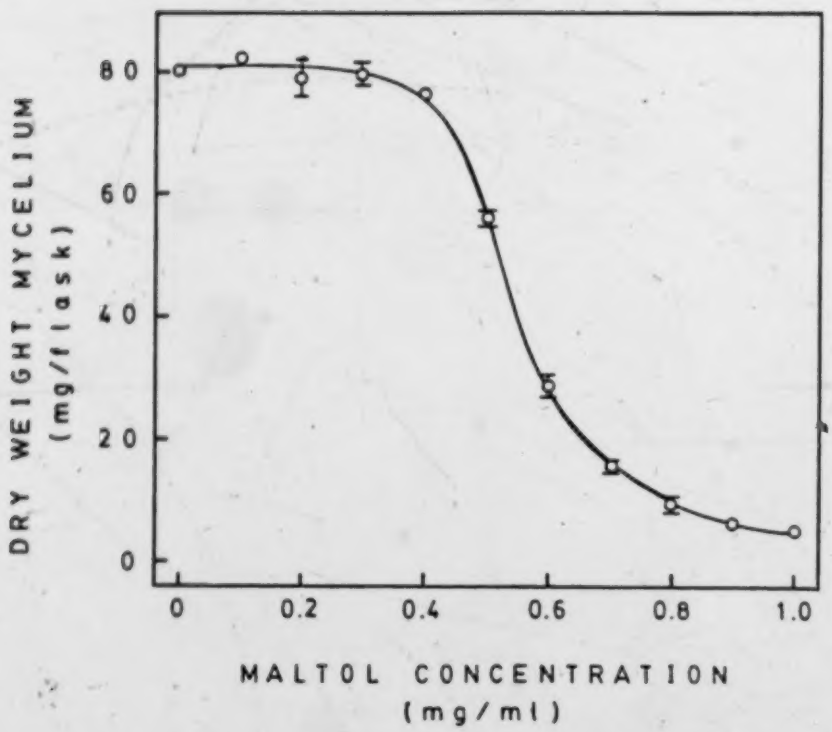

DRY WEIGHT MYCELIUM
(mg/flask)
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
MALTOL CONCENTRATION
(mg/ml)

Table 3. The activity of maltol against the mycelial growth of various wood decay fungi in submerged culture.

| Organism name | Isolate number* | Dry weight as % of control |
|---|---|---|
| Ceratocystis ulmi | C-645 | 50 |
| Fomes annosus | C-297 | 26 |
| Fomes annosus | C-298 | 1 |
| Fomes ignarius | C-381 | 44 |
| Fomes ignarius | C-606 | 37 |
| Fomes pini | C-288 | 70 |
| Peniophora giganteae | C-199 | 60 |
| Peniophora giganteae | C-200 | 73 |
| Polyporus abietinus | C-194 | 12 |
| Polyporus abietinus | C-208 | 54 |
| Polyporus adustus | C-590 | 62 |
| Polyporus schweinitzii | C-44 | 47 |
| Scytalidium uredinicola | 4149 | 30 |

* Numbers prefixed by C are organisms obtained the Canadian Forestry Service.

Note: the values reported are the mean of cultures containing 0.55 mg/mL maltol, as a percent of control cultures. All cultures were in duplicate.

and resistant systems for physiological studies on the activity of maltol. Considerable variation in MIC is known to arise from inoculum size (Sukroongreung et al. 1984), therefore a statistical design was used to circumvent this difficulty, rather than preparing inocula of a standard cell number for each isolate.

It was found that the growth of all test organisms at 48 hours incubation was completely inhibited by 4 mg/mL maltol, and none were inhibited by 0.5 mg/mL. The MIC for each isolate was determined (data presented in Table 4), and the distribution of MIC among the isolates is shown in Fig. 44. The distribution of MIC between repeats with selected isolates showed no significant difference from the distribution of MIC between isolates. For comparison, paired inoculations of Y237 and Y343 were made on the standard plates, and the paired MIC values obtained were analyzed by the sign test to determine if either strain showed an MIC greater than the other with statistically significant frequency. Y237 and Y343 were selected for the experiment because these two strains were found to have MIC values of 1.0 mg/mL and 4.0 mg/mL respectively in the original screening. The frequency distribution of MIC for the two strains is shown in Fig. 45. While there is some indication that a difference exists, no statistically significant difference could be detected. Whatever small differences may exist between the yeast isolates, none of

Table 4. MIC of maltol against 46 yeast isolates.

| Yeast | Strain No.* | MIC (mg/mL) |
|---|---|---|
| Candida parapsilosis | Y383 | 4 |
| Candida pelliculosa | Y166 | 2 |
| Cryptococcus albicans | Y455 | 1 |
| Debaryomyces kloecheri | Y155 | 4 |
| Endomycopsis capsularis | Y387 | 4 |
| Hansenula beijerinckii | Y229 | 2 |
| Hansenula canadensis | Y212 | 4 |
| Hansenula glucozyma | Y208 | 2 |
| Hansenula subpelliculosa | Y192 | 2 |
| Hansenula wingei | Y303 | 4 |
| Kluyveromyces fragilis | Y 44 | 2 |
| Kluyveromyces fragilis | Y 54 | 2 |
| Kluyveromyces marxianus | Y533 | 2 |
| Pichia angophorae | Y439 | 2 |
| Pichia bovis | Y426 | 2 |
| Pichia etchellsii | Y443 | 2 |
| Pichia fermentans | Y422 | 2 |
| Pichia haplophila | Y420 | 4 |
| Pichia kluyveri | Y425 | 4 |
| Pichia ohmeri | Y307 | 2 |
| Pichia orientalis | Y441 | 2 |
| Pichia pastoris | Y437 | 1 |
| Pichia pseudopolymorpha | Y423 | 2 |
| Pichia sargentensis | Y224 | 2 |
| Pichia spartinae | Y464 | 2 |
| Pichia toletana | Y444 | 2 |
| Pichia trehalophila | Y435 | 2 |
| Rhodotorula minuta | Y450 | 1 |
| Saccharomyces bayanus | Y160 | 2 |

Continued on following page

Table 4-Continued

| Yeast | Strain No.* | MIC (mg/mL) |
|---|---|---|
| Saccharomyces cerevisiae | Y175 | 4 |
| Saccharomyces chevalieri | Y281 | 4 |
| Saccharomyces coreanus | Y360 | 4 |
| Saccharomyces dairensis | Y372 | 2 |
| Saccharomyces diastaticus | Y285 | 4 |
| Saccharomyces fructuum | Y171 | 2 |
| Saccharomyces hienipiensis | Y343 | 4 |
| Saccharomyces lactis | Y144 | 2 |
| Saccharomyces logos | Y156 | 4 |
| Schwanniomyces alluvius | Y421 | 2 |
| Sporobolomyces odorus | Y400 | 1 |
| Sporobolomyces salmonicolor | Y399 | 2 |
| Torulopsis bombicola | Y445 | 1 |
| Torulopsis magnoliae | Y237 | 1 |
| Torulopsis schatavii | Y393 | 2 |
| Torulopsis sphaerica | Y165 | 1 |
| Torulopsis wickerhamii | Y311 | 2 |

* Yeast strain numbers are from the Prairie Regional Laboratory, Saskatoon, Canada.

the test organisms were found to be particularly sensitive nor resistant to the extent that the effect could be exploited for comparative studies on the activity of maltol.

Figure 44. The distribution of MIC of maltol between yeast isolates.

PDA plates containing the indicated concentration of maltol were inoculated with each yeast isolate in Table 5. A single stab was used as inoculum, and MIC was recorded as the concentration at which there was no apparent growth after 48 hours.

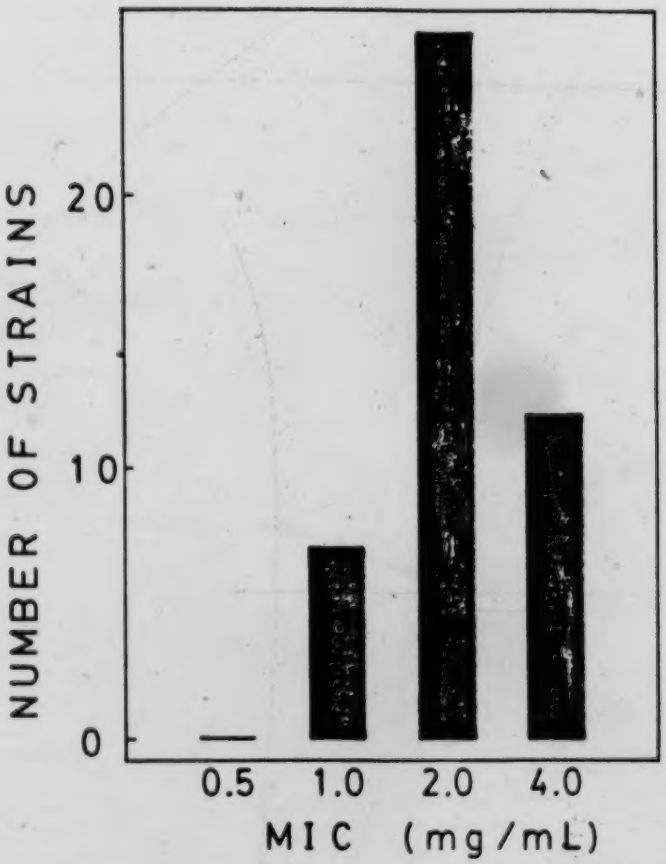
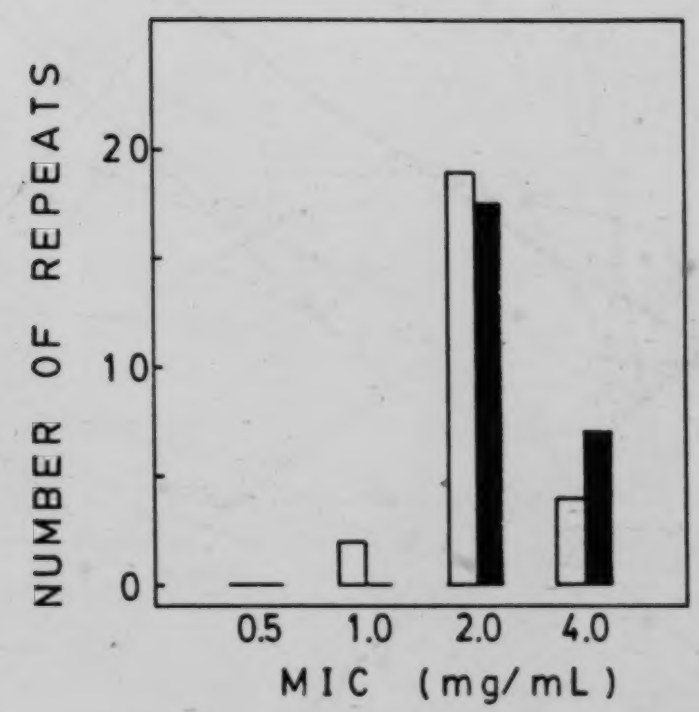

NUMBER OF STRAINS
20
10
0
0.5
1.0
2.0
4.0
MIC (mg/mL)

Figure 45. A comparison of the MIC frequency distribution for two yeast isolates.

For Y343 (Saccharomyces hienipiensis) and Y237 (Torulopsis magniliae), 25 repeat MIC determinations were made. The frequency distribution for Y237 is given by the open bars, and Y343 by the solid bars.


NUMBER OF REPEATS
20
10
0
0.5
1.0
2.0
4.0
MIC (mg/mL)

In Vivo Protection of Pine Seedlings with Maltol

The purpose of this experiment was to determine if sufficient translocation of maltol within the pine seedlings occurred to provide systemic protection, and to determine what amount of maltol was required to prevent colonization of the host tissue by E. harknessii. The appearance of a typical seedling and the extent of its root system at the age of inoculation is shown in Fig. 46. For each of the six trays of seedlings used, the number of plants showing signs of an active infection was recorded as a percent of the total number of plants given the same treatment. The data was analyzed as a simple randomized design, having 5 treatments (maltol doses) in each of the six blocks (trays). By analysis of variance, it was determined that the least significant difference between treatments was 16.3% at the 99% level of confidence. The mean percent infection for each treatment is given in Table 5. With a single application of 20 mg maltol per cavity, the percent of seedlings showing signs of infection was reduced to 49% from the control value of 92%, which is statistically a highly significant difference. Lesser doses of maltol, however, provided no apparent protection of the seedlings. Maltol had no observable effect on the plants with any of the amounts applied. It was not determined whether maltol treatment reduced percent germination of E. harknessii spores on the host, or if host penetration and colonization

Figure 46. Photograph of a typical lodgepole pine seedling at the age when inoculated with E. harknessii, showing the extent of its root system.

Lodgepole pine seeds were planted in peat moss with biweekly application of 20-20-20 fertilizer. The seedlings were grown at 20°C under natural light, supplemented by 400 W sodium lamps to give a minimum 12 hour photoperiod. The seedling is shown 2 months after planting; magnification is 0.83 x.

was aborted.

Table 5. The effect of maltol on pine seedling susceptibility to infection by *E. harknessii*

| maltol dose (mg/cavity) | percent of plants showing signs of infection |
|---|---|
| 20 | 49 |
| 2 | 93 |
| 0.2 | 92 |
| 0.02 | 90 |
| control | 92 |

Note: the least significant difference at the 99% level of confidence was 16.3 percent of plants showing signs of infection.

Presence of Maltol in Lodgepole Pine

Maltol was tentatively identified in samples of lodgepole pine needles. The filtrate taken from needles homogenized in water (acidified to pH 3 with HCl) was extracted with chloroform. The chloroform extract was analyzed by gas chromatography, and a peak having a retention time corresponding to a maltol standard was found. The extract was fractionated by preparative TLC over silica gel, and materials having the same $R_f$ as a maltol standard were recovered. This preparation was partially purified by column chromatography with Sephadex LH20 (as described in the inhibitor purification procedure, column IV). A band having the retention volume and reddish color characteristic of maltol was recovered; GC analysis and UV absorbance scan of this preparation indicated the presence of maltol. Further chemical characterization was not obtained. The amount of maltol recovered from the sample was estimated to be 13 μg/g of pine needles. Considerable variation between samples collected at various locations in Alberta was observed, some samples showing no detectable maltol.

## V. DISCUSSION

### Culture conditions and Inhibitor Production

There were two principal reasons in this project for investigating culture reproducibility. First was the development of a procedure for the isolation of the antifungal metabolite from culture extracts: the knowledge that the starting material, concentrated culture extract, was of consistent composition greatly aided progress in isolating and purifying the metabolites. Second, if S. uredinicola is to be used as a biocontrol agent, strain improvement and large scale production will require knowledge of the organism's growth characteristics and physiology. The growth kinetics of S. uredinicola in submerged culture was therefore considered pivotal, and is more fully described later in this section.

From initial observations, it became apparent that variation between cultures grown on different occasions exceeded that of cultures generated at the same time from a single inoculum preparation. The homogenizing and dispensing of inoculum were eliminated as major sources of error, and attention was then focussed on inoculum preparation. The standard scale-up procedure described in Materials and Methods was designed to ensure that cultures were inoculated with a specific mass of mycelium taken as close as possible to the same growth phase on each occasion. Size and age of inoculum were controlled because their

effect on the growth of fungi has been well documented (Meyrath 1962, Meyrath and Suchanek 1972, Taber 1957, Ward and Colotelo 1960, Metz and Kossen 1977).

Since the time course of inhibitor production was unknown, the cultures from which the inhibitor was isolated and identified were harvested when the cultures were entering stationary phase (after 10 days incubation). If the peak concentration of inhibitor was associated with either growth or stationary phases, it was anticipated that variation within the group of flasks harvested would allow for its detection. After maltol was identified as a sporostatic metabolite of S. uredinicola, its production could be followed. It was found that a peak concentration of maltol occurred at the end of the growth phase. The use of an arbitrary, standard culture procedure may also have selected for the isolation of maltol over other sporostatic substances. The production of maltol by S. uredinicola within Endocronartium galls was not investigated.

The production of maltol may be a significant aspect in the parasitic action of S. uredinicola on E. harknessii; therefore, it was of interest to learn what factors affected or controlled the amount of maltol produced. Large inocula gave the earliest peak maltol concentration, while the smallest inocula showed no significant production of maltol (Fig. 17). The largest inoculum produced cultures with maltol concentrations which were high, but persisted for the

shortest period of time. Variation in the time period during which high maltol concentration occurred contributed to the variance in maltol concentration recorded for replicate cultures. Cultures growing at a slower rate, similar to that of Fig. 17B, produced lower concentrations of maltol, but it persisted longer in the growth medium. For this reason, the slower-growing cultures may be preferable for studying the factors affecting maltol synthesis.

The concentrations of maltol produced in the defined medium used in this study were substantially lower than in the complex medium. However, isolation and quantitation of maltol was more difficult from the complex mixtures obtained by extracting malt extract broth cultures than defined medium extracts. Studies on the biosynthesis of maltol would be greatly aided by using the much cleaner extracts obtained from the defined medium. The highest concentration of maltol observed in defined medium was only about 1 µg/mL, compared to 50 µg/mL in malt extract broth. Lack of pH control in the synthetic medium might be a reason for low production.

Maltol does not appear to have been previously described as a microbial metabolite, but several other -pyranones have been (Turner 1971, Sato *et al*. 1969). One related compound, kojic acid (2-hydroxy-5-hydroxymethyl-4H-pyran-4-one), is believed to

be synthesized directly from glucose (Arnstein and Bentley 1956). Yields of Kojic acid from glucose as high as 50% have been reported (Turner 1971). For this reason, glucose was selected as the carbon source for maltol production in the synthetic medium. It is not known if other substrates would give greater yields.

Inhibitor Purification and Isolation

Culture extracts were first analyzed on silica gel thin layers to determine the distribution and mobility of the inhibitory activity. This was necessary to show that the solvent systems which gave good resolution of the less polar fractions were also moving all of the inhibitory activity from the origin. On thin layers and in columns of silica gel, a large portion of the culture extract remained at the origin during elution with chloroform or chloroform:methanol. The bioassay of thin layer fractions (Fig. 4) showed that all of the inhibitory activity was mobile over silica gel using the mixed solvent system. Compared to chloroform alone, the more polar mixture increased the mobility of the metabolites approximately 2-fold. For silica gel column chromatography, chloroform was used to give greater differences in retention volume between the metabolites, hence giving greater resolution.

The broad distribution of inhibitory activity on thin layers resulted from the large sample applied, uneven banding, and possibly from the existence of more than one sporostatic substance. Another distinct activity was found from the materials recovered at $R_f$ 0.2 to 0.3; they caused multiple germ tube formation by E. harknessii spores. E. harknessii normally produces only one germ tube (Powell and Morf 1966). It is possible that this material reduces the inoculum potential of hyperparasitized galls, but the effect

has not been reported in connection with spores taken from hyperparasitized galls. The directive of this part of the project was to isolate a germination or growth inhibiting substance; therefore, the isolation of the compound responsible for multiple germ tube formation was not pursued. If identified, this substance may be useful in studying the mechanism of germination.

The most abundant and readily obtained metabolite from the culture extracts did not have any sporostatic activity under the conditions used. This substance was obtained by precipitation from either methanol-water or chloroform-hexanes; it is also obtained in relatively pure form in the early fractions from silica gel column chromatography. Its $R_f$ on the analytical thin layers was approximately 0.6. Only partial characterization was obtained (mass spectrum m/e 206, UV[$H_2O$]$\lambda_{max}$ 242 nm). The only sporostatic activity detected in silica gel column fractions was associated with later, mixed fractions containing mostly benzeneacetic acid and maltol. Subsequent purification steps indicated that maltol was the only inhibitory substance present. Methanol washings of the silica gel column after chromatography did not contain any sporostatic substances, indicating that all of the inhibitory substances had been eluted.

The antifungal compound scytalidin was produced by S. album under the culture conditions used for S. uredinicola

metabolite production used in this project. Upon concentration of S. album culture filtrate, a white precipitate formed. The precipitate was collected by filtration, and crystallized from methanol. Chromatography, bioassay, and UV scanning results were identical to an authentic sample of scytalidin. No production of scytalidin occurred in S. uredinicola cultures.

Growth Kinetics of S. uredinicola

If S. uredinicola mycelium is to be produced for use as a biocontrol agent or as a source of useful metabolites, accurate prediction of culture development, regarding growth rate and mycelial morphology, is desirable. Information was gathered on both hyphal growth and culture growth, in order to establish a model that is consistent with both. The desire for such a model is twofold: prediction of culture growth from early development would be useful because specific growth rate is highly variable; mycelial morphology is related to culture growth rate.

Observations of hyphal growth from S. uredinicola colony margins and spore outgrowth yielded data consistent with other fungal systems (Butler 1961, Fiddy and Trinci 1976B), with some minor differences. In the spore outgrowth data shown in Fig. 19, the maximum elongation rate of the germ tube was established before branching occurred. The main axis and the branches formed appeared to have equal rates of extension which were achieved without a noticeable lag time. In most spore outgrowth observations, the organization was less apparent than that presented in Fig. 19. Branching often occurred sooner after germination, and the rates of elongation were less than maximal while branching continued.

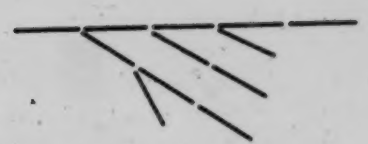
At colony margins, mature hyphae elongated at constant rates with septum formation at highly conserved intervals.

Branch formation did not appear to be correlated with septum formation as it is in other systems (Fiddy and Trinci 1976B, Butler 1961). A heirarchical organization of branch size and extension rate was not readily apparent; however, the data collected were insufficient to test for this possibility. It is known that a minimum length of hyphae is required to support maximal extension rate (Zalokar 1959). Accordingly, the extension rate of a S. uredinicola hyphae with a branch forming near the apex is slower than the maximum rate. This effect has been described as the peripheral growth zone by Fiddy and Trinci (1976A, 1976B).

It is convenient to depict hyphal growth as occurring by the idealized pattern shown in Fig. 47. Each segment represents the mean intercalary compartment length. This is essentially the same as Butler's idealized diagram of the branching system in Merulius lacrymans, without tendril hyphae and a hierarchy of branches (Butler 1958). The diagrams depict growth such that each tip elongates at the same constant rate. The formation of a branch is shown such that each tip is supported by a minimum length of hypha equal to an intercalary compartment length (earlier branching would result in reduced extension rates). The schematic diagrams show the mean length of each apical compartment as 1.5 times an intercalary compartment length, which approximates experimental observations in this study and elsewhere (Fiddy and Trinci 1976A).

Figure 47. A hypothetical model of the growth of a hyphal branching system.

Each segment represents the mean length of hypha between branches. Growth is depicted such that branching does not occur until each tip is supported by a minimal length of hypha (one segment). All tips are elongating at the same, linear rate. The units of time are defined as the time required for a tip to extend through a distance of 1 segment. Segments are used as the units for hyphal length.

| Units of Time | Diagram | Total Length |
|---|---|---|
| 1 |  | 1 |
| 2 |  | 2 |
| 3 | | 4 |
| 4 | | 7 |
| 5 |  | 12 |
| 6 | | 20 |

In Fig. 47, units of time are given as the time required for tip extension through a distance of one segment. The total length of hyphae at each time interval is given. This series of numbers may also be generated by equation 3 where L represents total hyphal length at the time indicated by the subscript, and t represents units of time.

$$L_t = L_{t-1} + L_{t-2} + 1 \qquad (3)$$

Equation 3 is not readily differentiable, and is not of any practical value because the segment length and units of time would be difficult to determine. However, a comparison of the function $L_t$ to the established growth models is informative. A typical culture undergoes about a 50-fold increase in mycelial mass from the time of inoculation to stationary phase; therefore, the growth of $L_t$ through 7 whole-number time intervals is used. Comparing the function $L_t$ to the curves described elsewhere (Emerson 1950, Carroad and Wilke 1977), it is apparent that the experimental best-fit equation of Carroad and Wilke (1977) most closely approximates the $L_t$ function. The best fit equation of Carroad and Wilke is in the form of equation 2 with $B \pm = 0.918 \pm 0.090$. A closer approximation of $L_t$ can be made using equation 2 with $B = 0.833$ (growth by a $6^{th}$ root relationship), which lies within 1 standard deviation of

Carroad and Wilke's best-fit B. A summary of the coefficients of determination for the $L_t$ function fitted to each equation is given in table 6.

Table 6. A comparison of four growth functions fitted to a hypothetical model ($L_t$).

| equation | $r^2$ |
|---|---|
| $dX/dt = X$ | 0.994 |
| $dX/dt = A\ X^{2/3}$ | 0.986 |
| $dX/dt = A\ X^{0.918}$ | 0.995 |
| $dX/dt = A\ X^{0.833}$ | 1.000 |

note: $r^2$ is the coefficient of determination, which gives the proportion of variance explained by the relationship between X and t.

As pointed out by Carroad and Wilke (1977), for the purpose of fitting a curve to experimental data "...it does not make a great deal of difference which model one chooses." However, the $L_t$ function based on an approximation of the structural basis of growth, and the models based on equation 2, each differ from the exponential

model in that the latter is independant of inoculum size. The effect of inoculum size on growth rates is well documented (Meyrath and Suchanek 1972). The exponential growth model is based on the existence of an ill-defined duplicating unit as the structural basis of growth. This model does not permit different rates of growth for equivalent mycelial masses. In contrast, when considering the hyphal branching system as the structural basis of growth, the effect of inoculum size becomes evident. Both the number of growing apices and morphology may be expected to differ as shown in Fig. 27. These effects are evident in submerged culture by different specific growth rates and hyphal agglomeration (pellet formation). The increasing time interval between growth curves shown in Fig. 35 serves to illustrate this point: two equal masses of mycelium raised from different inoculum sizes have different specific growth rates. General equation 2 gives a straight line relationship between the time interval between the curves and time. The relationship is given by equation 4 (the derivation of equation 4 from equation 2 is given in appendix B).

$$Y = (A^{1/\alpha} - 1)\, Z + K \qquad (4)$$

In equation 4, Y represents the time interval between curves at the culture mass on both curves equal to the mass

of the large inoculum culture at time = Z. Z represents the age of the large inoculum culture, A the mycelial mass of the large inoculum divided by the mass of the small inoculum, and K the value of Y at the time of inoculation. The value of c is determined from experimental values of Y.

To represent batch culture, the experimental value of c is used in equation 5, which is differentiated to give the culture growth rate in the form of general equation 2. In equation 5, D is a constant, t represents culture age, and c is found experimentally from equation 4. X represents

$$X = D\, t^{c} + i \qquad (5)$$

total mycelial mass, and i the mass of the inoculum.

The value of B determined experimentally for S. uredinicola cultures (Fig. 35) by measuring Y was found to be 0.826. This result is close to the $6^{th}$ root function (B = 0.833) predicted by the hypothetical $L_t$ function taken from the idealized model of hyphal growth (Fig. 47). The early growth phase of S. uredinicola 4149 in malt extract broth was fitted to the logarithmic, cube root, and $6^{th}$ root functions. The experimental data most closely matched the cube root function (Fig. 26). The value of the exponent B may likely vary between that determined by comparing two curves and that obtained by fitting a single curve, because the general equation 2 is only an approximation of an

unknown function defined by the structural basis of growth. Although it may be possible to collect sufficient data on hyphal growth to construct an accurate formula, it would likely be in a form too inconvenient for practical purposes. Of the established general formulae (equations 1 and 2), equation 2 is preferable because it accounts for the inoculum size effect (the constant A is in direct proportion to inoculum size). Moreover, promotion of pellet formation by reduced inoculum size is therefore directly associated with the culture growth rate. Mycelial morphology may be a significant variable if mycelium is to be tested as a biocontrol agent. Furthermore, separation of loose mycelium from the medium by filtration is sometimes difficult and slow, whereas the compact pellets are easily and rapidly separated. It also appeared that general equation 2 was more consistent with the structural basis of fungal growth than was the exponential growth model.

## Carbon and Nitrogen Utilization in S. uredinicola Cultures

The manipulation of carbon source and nitrogen source requirements were of interest in this project for three reasons. First, the efficacy of S. uredinicola mycelium if used as a biological control agent may be affected by its physiological state. Second, the quantity of residual carbohydrate in the medium during stationary phase may affect the production of useful fungitoxic metabolites. Third, some control over the economic coefficient of mycelium or metabolite production may be gained.

Carbon and nitrogen requirements of fungi in submerged cultures are known to be affected by inoculum size (Meyrath and McIntosh 1963, McIntosh and Meyrath 1963). The requirements also change with culture age, and it has been suggested that the mycelium in a small-inoculum culture is affected by an ageing process during the time required to produce the mass equal to a large inoculum culture (Meyrath and McIntosh 1963, McIntosh and Meyrath 1963). However, the trends observed in specific nutrient utilization may be complicated by the measurement of growth used. A large portion of mycelial dry weight increase may occur after the end of replicatory growth, due to cell wall thickening and lipid accumulation. This was shown in Penicillium urticae by the divergence of dry weight and total cell protein at the end of growth phase (Grootwassink and Gaucher 1980). For the present purposes, the ratio of carbon source to

nitrogen source utilized is used to reveal trends due to inoculum size, culture age, and mycelial mass. The ratio gives a physiological measurement which is unaffected by the uncontrolled variables associated with measuring growth.

In complex medium the growth rate, mycelial yield, and mycelial morphology were affected by inoculum size. The possibility that the effect of inoculum size on yield might involve altered carbon and nitrogen requirement was investigated by measuring glucose, ammonium, and nitrate levels in defined medium cultures. In the defined medium, nitrogen did not appear to be limiting. After dry weight ceased to accumulate residual ammonium concentration was about 25%, and utilization of nitrate had not occurred. Some residual glucose was also present; however, it was not certain if the concentration was growth limiting. Inoculum size affected growth rate and mycelial morphology in the defined medium, but the effect on mycelial yield was not significant. This may have been the consequence of growth being limited by different factors in the defined medium than in the complex medium.

The ratio of glucose to nitrogen removed from the medium increased with inoculum size when measured at a single culture age. The effect was shown for a time point during unrestricted growth and at the end of the growth phase, indicating that the trend was not associated with metabollic phasing in the cultures. By interpolation, the

carbon-nitrogen utilization ratio was estimated for each inoculum size at a culture mass of 0.2 mg dry weight (data not presented). No apparent trends in the carbon-nitrogen utilization ratio with inoculum size occurred in this case. From these data, it appeared that the ratio of carbon to nitrogen used was specific for culture mass, and not culture age.

To elaborate on the above observations, the carbon-nitrogen ratio was determined along two growth curves having a 10-fold difference in inoculum size. The carbon-nitrogen ratio of each curve increased with age and mycelial mass. However, with age the two curves were separated, but with mass the two inoculum sizes were indistinguishable (Fig. 34). As before, it appeared that the carbon-nitrogen utilization ratio is specific for mycelial mass. The independance of the ratio from ageing of mycelium is also evident by the ratio approaching a maximum value with age, paralleling culture growth. The ratio is apparently in direct proportion to mycelial mass. A number of other variables such as oxygen limitation, vitamin and trace element requirements, and substrate concentration change with or regulate growth, but the effects of these variables were not investigated.

Some significant trends in carbon and nitrogen source requirements have been revealed. However, to take full advantage of this knowledge, these trends must be considered

in relation to specific nutrient uptake, growth kinetics, and the goal of the fermentation. When glucose and ammonium utilization per mass of mycelium (as dry weight) was calculated, specific glucose utilization remained relatively constant throughout all phases of growth, and specific ammonium utilization declined with culture age. No significant differences in these values between inoculum sizes was noted. If the goal is to produce biomass for use as a biocontrol agent, a large inoculum and an early harvest gives a shorter fermentation time and reduces the amount of nitrogen source required to produce a given mass of mycelium. These advantages, however, must be considered in relation to the quality of the mycelium which results (i.e. pellet formation and size). Similarly, if the production of maltol or other metabolites is of interest, the net requirement of substrate and growth rate might be manipulated for economic advantage.

Maltol production in defined medium was reexamined after the carbon and nitrogen requirements of the S. uredinicola cultures had been established. The amount of glucose used was selected to give about 50% residual glucose at the time of nitrogen depletion. Although the concentration of phosphate was also increased over that used in earlier experiments, the final pH of the medium fell to 2.7. The time course of maltol production was similar to that observed in malt extract broth; however, the maximum

concentration was only 0.4 µg/mL.

Biological Effects of Maltol

E. harknessii spore germination was inhibited by concentrations of maltol much lower than that required to reduce mycelial growth of most other fungi. The difference may be partly due to the methods used. In the spore germination assay, the proportion of the population of spores affected by maltol is directly measured. The yield of mycelium is not a direct measure; the growth kinetics and medium components may have influenced the apparent activity of maltol. Germ tube length did not appear to be affected by the presence of maltol, even when inhibition was greater than 90%. The mode of action of maltol as a sporostatic, fungitoxic, or antibiotic agent is not known. Since maltol is an inhibitor of glyoxalase I, structural analogues which also inhibit glyoxalase I were tested as germination inhibitors. The effects of the analogues on germination did not compare well with the activity of maltol. Chelation of calcium ion by maltol as a mechanism of inhibition was tested by supplying additional calcium, but the inhibition due to maltol was not reduced. It was found that glutamic acid increased the inhibitory activity of maltol, but alone had no effect on germination: this observation was contradictory to the antibacterial activity (Banerjee et al. 1980).

Maltol is considerably less potent than the fungicides currently available. The $ED_{100}$'s for Ferbam, Ziram, and

Zineb range from 0.1 to 10 µg/mL, depending on the test organism (Owens 1969). About 550 µg/mL maltol is required to approach 100% inhibition of E. harknessii germination, and it has variable activity against other fungi (Table 4). The chemical properties of maltol indicated that it may be able to translocate within plants and provide systemic protection; this has not been achieved with conventional fungicides or with antibiotics used against pine stem rusts (Merril and Kistler 1976, Hinds et al. 1966). Whether maltol will be useful as a fungicide is contingent upon its systemic activity and cost effectiveness.

The ability of maltol to translocate within pine seedlings to the site of infection was tested by applying aqueous maltol to the soil. By applying 20 mg of maltol per soil cavity two days prior to inoculation with E. harknessii spores, the number of plants infected was reduced to 53% of the control. it was not determined if infection was reduced through inhibition of spore germination or protection from penetration into the host tissue. Protection of the seedlings by uptake of maltol through the roots indicated that maltol was able to penetrate through the host tissue to the site of infection. To assess the feasibility of using maltol as a control agent, optimal timing and method of application must be established. The effect of maltol on an active infection should be investigated next, to determine if sporulation and gall development can be suppressed.

That maltol is a constituent of many plants raises some intriguing possibilities. Natural host resistance is known to occur (Hutchenson 1935), therefore a comparison of maltol content between resistant and susceptible trees would be a logical undertaking. Preliminary data (not presented) have indicated a possible relationship; however, a very large sample size will be required to demonstrate the significance of this finding. The classification of trees as resistant or susceptible, and concomitant variables affecting infection rates will likely be significant sources of error. Plant selection for trees having high maltol content is another avenue to be explored.

## VI. CONCLUSIONS

The compound maltol was found to be a metabolite of S. uredinicola, and it is presumably responsible for part of the antagonism of S. uredinicola against E. harknessii in pine stem galls. Maltol is systemically active in lodgepole pine seedlings, giving significant protection against infection by E. harknessii. Although maltol is not a highly potent fungicide, its ability to permeate the host tissue may be an advantage over presently available fungicides. Conventional fungicides prevent infection, but do not penetrate host tissue to inactivate galls or abort incipient infections in nursery stock used for outplanting. Further investigation is therefore warranted to determine whether maltol is a solution to these problems.

S. uredinicola growth rate and morphology in submerged culture is affected by inoculum size. Trends in the ratio of carbon to nitrogen used by a culture are linked to mycelial mass and are therefore influenced by inoculum size. Mycelial inoculum is difficult to standardize because equal masses from different cultures may have different morphologies and growth rates. These variables present difficulties in several areas of experimentation. Reproducible cultures for screening of fungal strains are difficult to obtain; these experiments must be designed to identify error due to growth rate, morphology, and nutritional requirements where they might affect data

interpretation. Control over mycelial morphology is desirable for testing S. uredinocola as a biocontrol agent, and as a consideration in separating the biomass from the medium. Predictable culture development is needed for studying metabolite production.

The general equation $dX/dt = A X^B$ where X is mycelial mass, A is a constant, and the exponent B is determined experimentally gave the best approximation of the unrestricted growth phase. The advantage of representing batch culture by this formula rather than the commonly used logarithmic model is that the constant A can be varied with inoculum size, accounting for different culture growth rates. The value of A is in direct proportion to inoculum size when inocula are taken from the same preparation (i.e. culture homogenate). Since inocula are difficult to reproduce, the virtual value of A may be calculated from early values of $dX/dt$ and $X^B$ in a culture, thus enabling prediction of culture development. Determination of appropriate harvest time for desired mycelial morphology, net carbon and nitrogen utilization, and optimal metabolite production might be possible.

The structural basis of mycelial growth may be conceptualized from the known and measurable properties of hyphal branching systems. The overt characteristics reported elsewhere, linear rates of extension, conserved intercalary lengths, and net acropetal succession of branch

formation are observed with S. uredinicola. The properties of culture growth (influence of inoculum size on growth rate and morphological development) observed experimentally are compatable with this concept (Fig. 47). Although it is not practical to gather sufficient data to construct an exact model, it is significant that the observable features of culture growth are incompatable with the existence of a duplicating unit of mycelial growth.

That maltol is organic, biodegradable, and compartively non-toxic to animals are ecological advantages over some fungicides being used. The cost effectiveness cannot be calculated until further experimentation is completed. The mode of action of maltol against fungi is not known; investigation in this area might lead to a more effective agent. Since maltol is a natural constituent of many pines, the development of plant strains having high maltol content might provide rust resistant nursery stock. Another approach to western gall rust suppression is the use of S. uredinicola as a biocontrol agent. The results of this study indicate that the development of a high maltol producing strain of S. uredinicola might be more effective. Maltol is available from natural sources (tree bark) and chemical syntheses; production by fungal fermentation is not feasible (less than 0.01% yield from glucose was obtained in synthetic medium).

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## APPENDIX A

### Maltol: A Literature Review

The compound maltol (3-hydroxy-2-methyl-4H-pyran-4-one) was first isolated from the bark of Larch trees (Pinus larix), and has since been isolated from a variety of plant sources as shown in Table 7. Maltol is also a Maillard reaction product (browning reactions); consequently it is found in many food materials that contain carbohydrates and proteins and have been subjected to high temperatures (Banerjee et al. 1979). Approved for food use by the FDA, and listed by the Council of Europe with an ADI of 1 mg/kg, maltol is commonly added to foodstuffs as a flavor enhancer, as well as to soaps, detergents, creams, lotions, and perfumes for fragrance properties (Opdyke 1975). A weak antimicrobial property of maltol has also been described (Banerjee et al. 1979, Fitzgerald 1953, Wolf and Westveer 1950).

The first chemical synthesis of maltol was reported by Spielman and Friefelder (1947). In this synthesis, pyromeconic acid (3-hydroxy-4H-pyran-4-one) is methylated at the 2 position by forming a mannich base with piperidine and formaldehyde, followed by hydrogenation over palladium. The yield of maltol was very low. Maltol is more readily obtained by a patented process involving aqueous extraction of maltol-containing tree bark, followed by extraction of the aqueous solution with chloroform. The crude maltol is

Table 7. Plants from which maltol has been isolated.

| Plant | Reference |
|---|---|
| *Abies alba* | Opdyke 1975 |
| *Pinus larix* | Opdyke 1975 |
| *Abies mariesii* | Kihara 1983 |
| *Abies veitchii* | Kihara 1983 |
| *Abies homoleppis* | Kihara 1983 |
| *Abies leptolepis* | Kihara 1983 |
| *Tsuga diversifolia* | Kihara 1983 |
| *Chamaecyparis* | Kihara 1983 |
| *Evodiopanax innovance* | Kihara 1983 |
| *Rhododendron reticulatum* | Kihara 1983 |
| *Cercidiophyllum japonicum* | Kihara 1983 |
| *Clerodendron bungei* | Zhou 1982 |
| *Geranium macrorrhizum* | Ognyanov *et al*. 1975 |
| *Abies sibirica* | Tyukavkina *et al*. 1972 |
| *Helichrysum ramosissimum* | Lassak and Pinhey 1968 |
| *Passiflora incarnata* | Aoyagi *et al*. 1974 |
| *Enkianthus subsessilis* | Ogawa and Ogihara 1976 |
| *Panax ginseng* | Wei 1982 |

purified by azeotropic distillation with ethylene glycol, and recrystallized from water or alcohol (Aeintz et al. 1970). Syntheses of maltol from glucose (Shono et al. 1983) and from 1-(2-furyl)ethanol (Harada and Iwasake 1983) have also been reported. The five step synthesis from glucose may provide an 81% yield of maltol; the synthesis from 1-(2-furyl)ethanol is a 1-pot sequence yielding 75%.

The diverse plant sources from which maltol has been isolated include herbaceous plants as well as a variety of coniferous trees. In a survey of 35 species of Japanese conifers, it was noted that maltol was only detected in species which are characteristically found at sub-alpine elevations. These species (Abies mariesii, A. veitchii, A. homolepis, and Tsuga diversifolia) are also known to have high sugar content, presumably to prevent freezing. The authors reported levels of 0.5% to 3.5% in leaves, and speculate that the plants may be producing maltol biosynthetically from sugars (Takaishi et al. 1976).

Maltol was included in a series of α,β-unsaturated ketones that were tested for antimicrobial activity on the premise that conjugate addition to thiol groups of essential enzymes occurred. The antimicrobial effect of maltol was not lost by reduction of the α,β-unsaturation, thus indicating that the activity may not involve reaction with the thiol moity of proteins. Three bacterial and three fungal test organisms were used: 6 to 15 mg/mL maltol was

required to completely inhibit bacterial growth, whereas 2 to 4 mg/mL prevented fungal growth (Wolf and Westveer 1949). In an evaluation of various fungicides for use on moist hay, Schenk and Kennedy (1955) found that mold growth was reduced but not prevented completely by 1.2% maltol.

In a study of the effects of inhibitory agents against representative oral Lactobacilli, including several isolates of L. casei, Fitzgerald and Jordan (1953) found that maltol concentrations of up to 4 mg/mL failed to inhibit growth in tomato juice broth. Banerjee et al. (1979) later reported the inhibition of L. casei growth and lactic acid generation by only 0.5 mg/mL maltol; however, the authors also demonstrated reversal of the maltol effect by the addition of various amino acids and combinations of amino acids to the medium. They reported complete protection from the effect of maltol by the addition of glutamate to the medium.

Douglass and Nadvi (1979) selected a number of compounds for their resemblance to the enediol intermediate which is believed to occur in the glyoxalase-I-catalyzed conversion of the glutathione-methylglyoxal adduct to s-D-lactoylglutathione. These transition state analogues, which included maltol, were tested as inhibitors of yeast glyoxalase I. Non-competitive inhibition due to maltol was observed, and the $K_i$ was determined to be $3.94 \times 10^{-4}$ M (0.05 mg/mL). Although the function of the glyoxalase system is obscure, regulation of cell growth and protection

from α-ketoaldehyde toxicity has been considered (Oray and Norton 1980).

## APPENDIX B

### The Derivation of Equation 4

Two hypothetical growth curves are generated: new mycelial mass (X) for both are directly proportional to time raised to the exponent c ($t^c$). The inoculum for one curve ($X_a$) is A times greater than that of the other curve ($X_b$). Therefore, for each time point $X_b = AX_a$. The time interval between $X_a$ and $X_b$ when $X_a = X_b$ is defined by Y.

To obtain Y in terms of $t_a$:

$$X_a = X_b$$

therefore $$At_a^c = t_b^c$$

and $$(A^{1/c})t_a = t_b$$

since $$Y = t_b - t_a$$

$$Y = ([A^{1/c}]t_a - t_a)$$

$$Y = (A^{1/c} - 1)t_a$$

To determine Y from total mycelial mass, rather than from new mycelial mass, the constant K must be added. K is the value of Y at the time of inoculation. To avoid confusion with other formulae in the text, the age of the large-inoculum culture is redefined as Z. This gives equation 4 as follows:

$$Y = (A^{1/c} - 1)Z + K \qquad (4)$$

## APPENDIX C

Microbiological Growth Media

1) Malt extract broth

Difco malt extract broth (20 g/L) was autoclaved at 20 psi for 15 minutes.

2) Glucose salts medium

The basal synthetic medium of Jennison et al. (1959) was used with some modifications. The concentrations of glucose, phosphate, and the nitrogen sources were varied for experimental purposes.

| medium component | quantity/L |
|---|---|
| glucose | varied |
| $NH_4NO_3$ or $NH_4Cl$ | varied |
| $KH_2PO_4/K_2HPO_4$* | varied |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g |
| KCl | 1.0 g |
| thiamine·HCl | 1.0 mg |
| $H_3BO_4$ | 2.0 mg |
| $CuSO_4 \cdot 5H_2O$ | 0.2 mg |
| $FeCl_2$ | 1.0 mg |
| $MnCl_2 \cdot 4H_2O$ | 0.2 mg |
| $NaMoO_4 \cdot 2H_2O$ | 0.2 mg |
| $ZnCl_2$ | 2.18 mg |

* Stock solutions of $KH_2PO_4$ and $K_2HPO_4$ were added in a ratio of 8.77 to 1.23 to give pH 6.0. the concentration of phosphate is given in the results section for each experiment.

3) Potato dextrose agar

Difco potato dextrose agar (PDA) was rehydrated and sterilized by autoclaving at 20 psi for 15 minutes. For plates to which maltol was added for yeast MIC determinations, aqueous maltol was combined with molten, concentrated PDA after autoclaving.

4) Glucose peptone yeast-extract agar

Glucose peptone yeast-extract agar (ATCC catalogue of strains 1982) was used for the maintenance of yeast cultures.

| medium component | quantity |
|---|---|
| glucose | 5.0 g |
| peptone | 5.0 g |
| yeast extract | 3.0 g |
| agar | 20.0 g |
| distilled water | 1.0 L |

5) Cereal agar

Cereal agar was used for the maintenance of Scytalidium and wood decay isolates.

| medium component | quantity |
|---|---|
| Pablum (Mead Johnson) | 10.0 g |
| Agar | 1.5 g |
| distilled water | 1.0 L |

## APPENDIX D

Glossary of Mycological Terms Used

Acropetal: Developing toward the apex.

Aecium: A structure consisting of binucleate hyphal cells which produce aeciospores.

Aecioid teliospore: Basidia-producing spores which resemble aeciospores (in the case of Endocronartium, basidiospores are not produced).

Arthroconidia (Arthrospore, Oidium): A spore resulting from the fragmentation of a hypha.

Autoecious: The ability of a fungus to complete its entire life cycle on a single host species.

Dematiaceous: Darkly pigmented.

Germ tube: The initial, hyphal protrusion from a germinating spore.

Heteroecious: The requirement of two host species for a fungus to complete its entire life cycle.

Hypha: The tubular filament which is the structural unit of fungi.

Intercalary: Interposed, as in the section of hypha between two branches.

Mycelium: The mass of hyphae constituting the body of the fungus.

Spermagonium: A structure containing small spore-like bodies which may function as spermatia.